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(54) Title: **TISSUE PROTECTIVE CYTOKINES FOR THE PROTECTION, RESTORATION, AND ENHANCEMENT OF RESPONSIVE CELLS, TISSUES AND ORGANS**

Capillaries of the human brain express very high levels of EPO receptor, as determined by immunohistochemistry using specific anti-EPO receptor antibodies. This provides a mechanism whereby EPO is able to penetrate into the brain from the systemic circulation, in spite of the blood brain barrier.



(57) Abstract: Methods and compositions are provided for treating a mammal having inflammation by protecting or enhancing a responsive cell, tissue, organ or body part exhibiting or associated with the inflammation, by systemic or local administration of a composition comprising a tissue protective cytokine. The invention also encompasses combination treatments comprising administering a composition comprising a tissue protective cytokine of the invention and administering at least one anti-inflammatory or least one immunomodulatory agent.

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**TISSUE PROTECTIVE CYTOKINES FOR THE PROTECTION, RESTORATION,  
AND ENHANCEMENT OF RESPONSIVE CELLS, TISSUES AND ORGANS**

[0001] CROSS-REFERENCE TO RELATED APPLICATIONS

[0002] This application claims priority to United States Patent Application No. 10/188,905, filed July 3, 2002, which is incorporated herein in its entirety.

[0003] BACKGROUND OF THE INVENTION

[0004] For many years, the only clear physiological role of erythropoietin had been its control of the production of red blood cells. Recently, several lines of evidence suggest that erythropoietin, as a member of the cytokine superfamily, performs other important physiologic functions which are mediated through interaction with the erythropoietin receptor (erythropoietin-R). These actions include mitogenesis, modulation of calcium influx into smooth muscle cells and neural cells, production of erythrocytes, hyperactivation of platelets, production of thrombocytes, and effects on intermediary metabolism. It is believed that erythropoietin provides compensatory responses that serve to improve hypoxic cellular microenvironment as well as modulate programmed cell death caused by metabolic stress. Although studies have established that erythropoietin injected intracranially protects neurons against hypoxic neuronal injury, intracranial administration is an impractical and unacceptable route of administration for therapeutic use, particularly for normal individuals. Furthermore, previous studies of anemic patients given erythropoietin have concluded that peripherally-administered erythropoietin is not transported into the brain (Marti *et al.*, 1997, *Kidney Int.* 51:416-8; Juul *et al.*, 1999, *Pediatr. Res.* 46:543-547; Buemi *et al.*, 2000, *Nephrol. Dial. Transplant.* 15:422-433).

[0005] Various modified forms of erythropoietin have been described with activities directed towards improving the erythropoietic activity of the molecule, such as those with altered amino acids at the carboxy terminus described in U.S. Patent 5,457,089 and in U.S. Patent 4,835,260; erythropoietin isoforms with various numbers of sialic acid residues per molecule, such as described in U.S. Patent 5,856,298; polypeptides described in U.S. Patent 4,703,008; agonists described in U.S. Patent 5,767,078; peptides which bind to the erythropoietin receptor as described in U.S. Patents 5,773,569 and 5,830,851; and small-molecule mimetics as described in U.S. Patent 5,835,382.

[0006] The present invention relates to tissue protective cytokines generated by the chemical modification of erythropoietin and their uses for protecting, maintaining, enhancing, or restoring erythropoietin-responsive cells and associated cells, tissues and organs *in situ* as well as *ex vivo*, and to delivery of a tissue protective cytokine across an endothelial cell barrier for the purpose of protecting and enhancing erythropoietin-responsive cells and associated cells, tissues and organs distal to the vasculature, or to carry associated molecules across an endothelial cell barrier.

[0007] BRIEF SUMMARY OF THE INVENTION

[0008] In one aspect, the present invention is directed to the use of tissue protective cytokines, chemically-modified erythropoietins that lack one or more aspects of erythropoietin's affect on the bone marrow, for the preparation of pharmaceutical compositions for protecting, maintaining, enhancing, or restoring the function or viability of responsive mammalian cells and their associated cells, tissues and organs. In one particular aspect, the responsive mammalian cells and their associated cells, tissues or organs are distal to the vasculature by virtue of a tight endothelial cell barrier. In another particular aspect, the cells, tissues, organs or other bodily parts are isolated from a mammalian body, such as those intended for transplant or reattachment. By way of non-limiting examples, the responsive cell or tissue may be neuronal, retinal, muscle, heart, lung, liver, kidney, small intestine, adrenal cortex, adrenal medulla, capillary endothelial, testes, ovary, pancreas, bone, skin or endometrial cells or tissue. Further, non-limiting examples of responsive cells include photoreceptor (rods and cones), ganglion, bipolar, horizontal, amacrine, Müller, myocardium, pace maker, sinoatrial node, sinus node, junction tissue, atrioventricular node, bundle of His, hepatocytes, stellate, Kupffer, mesangial, renal epithelial, tubular interstitial, goblet, intestinal gland (crypts), enteral, endocrine, glomerulosa, fasciculate, reticularis, chromaffin, pericyte, Leydig, Sertoli, sperm, Graffian follicle, primordial follicle, islets of Langerhans,  $\alpha$ -cells,  $\beta$ -cells,  $\gamma$ -cells, F-cells, osteoprogenitor, osteoclasts, osteoblasts, endometrial stroma, endometrial, stem and endothelial cells. These examples of responsive cells are merely illustrative. In one aspect, the responsive cell or its associated cells, tissues, or organs are not excitable cells, tissues, or organs, or do not predominantly comprise excitable cells or tissues. In a particular embodiment, the mammalian cell, tissue or organ for which an aforementioned tissue protective cytokine is used are those that have expended or will expend a period of time under at least one condition adverse to the viability of the cell, tissue or organ. Such conditions include traumatic *in-situ* hypoxia or

metabolic dysfunction, surgically-induced *in-situ* hypoxia or metabolic dysfunction, or *in-situ* toxin exposure; the latter may be associated with chemotherapy or radiation therapy. In one embodiment, the adverse conditions are a result of cardio-pulmonary bypass (heart-lung machine), as is used for certain surgical procedures.

[0009] The tissue protective cytokines herein are useful for the therapeutic or prophylactic treatment of human diseases of the CNS or peripheral nervous system which have primarily neurological or psychiatric symptoms, as well as ophthalmic diseases, cardiovascular diseases, cardiopulmonary diseases, respiratory diseases, kidney, urinary and reproductive diseases, gastrointestinal diseases and endocrine and metabolic abnormalities, and inflammation.

[0010] The invention is also directed to pharmaceutical compositions comprising particular tissue protective cytokines for administration to a mammalian animal, preferably a human. Such pharmaceutical compositions may be formulated for oral, intranasal, or parenteral administration, or in the form of a perfusate solution for maintaining the viability of cells, tissues or organs *ex vivo*.

[0011] Tissue protective cytokines useful for the aforementioned purposes and pharmaceutical compositions include erythropoietins that have been altered by at least one modification as compared to a native erythropoietin, and preferably as compared to native human erythropoietin. The at least one modification may be a modification of at least one amino acid of the erythropoietin molecule, or a modification of at least one carbohydrate of the erythropoietin molecule. Of course, tissue protective cytokine molecules useful for the purposes herein may have a plurality of modifications compared to a native molecule, such as multiple modifications of the amino acid portion of the molecule, multiple modifications of the carbohydrate portion of the molecule, or at least one modification of the amino acid portion of

the molecule and at least one modification of the carbohydrate portion of the molecule. The tissue protective cytokine molecule retains its ability of protecting, maintaining, enhancing or restoring the function or viability of responsive mammalian cells, yet one or more properties of the erythropoietin molecule unrelated to the aforementioned, desirable feature may be absent as compared to the native molecule. In a preferred embodiment, the tissue protective cytokine lacks erythropoietin's affects on the bone marrow, *i.e.*, increased hematocrit (erythropoiesis), vasoconstriction (high blood pressure), increased blood pressure, hyperactivation of platelets, pro-coagulant activities, and increased production of thrombocytes. More preferably, the tissue protective cytokines lack erythropoiesis; most preferably the tissue protective cytokines are devoid of all of erythropoietin's effects on the bone marrow.

[0012] By way of example, the tissue protective cytokine of the invention may be asialoerythropoietin. In another example, the tissue protective cytokine of the invention may be erythropoietin or asialoerythropoietin that has been reacted with one or more reagents that modify one or more amino groups of amino acid residues of native erythropoietin or asialoerythropoietin. In a preferred embodiment, the tissue protective cytokine is nonerythropoietic.

[0013] In one embodiment, the tissue protective cytokine is an erythropoietin that has no sialic acid moieties. In a preferred embodiment, the tissue protective cytokine is asialoerythropoietin, and most preferably, human asialoerythropoietin. In another embodiment, the tissue protective cytokine has 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or 13 sialic acid moieties. Such partially desialylated erythropoietins are referred to herein as hyposialoerythropoietins. They may be prepared by chemical or enzymatic modification of native erythropoietin, or may be obtained by expression in a system which either does not sialylate the molecule at all or only

partially sialylates the erythropoietin. The asialoerythropoietin and hyposialoerythropoietin of the invention are embraced regardless of the means by which the molecules are prepared.

[0014] In another preferred embodiment, the tissue protective cytokine comprises at least one or more modified lysine residues or a modification of the N-terminal amino group of the erythropoietin molecule, such modifications as those resulting from reaction of the lysine epsilon amino group or the N-terminal amino group with an amino-group-modifying agent or agents. The modified lysine residue or modified N-terminal amino group further may be chemically reduced. In one preferred embodiment, an erythropoietin is biotinylated, carbamylated, succinylated or acetylated at one or more lysine groups or at the N-terminus. In another preferred embodiment, the lysine is reacted with an aldehyde or reducing sugar to form an imine, which optionally is then stabilized by chemical reduction such as by using sodium cyanoborohydride to form an N-alkylated lysine residue such as glucitolyl lysine, or which in the case of reducing sugars may be stabilized by Amadori or Heyns rearrangement to form an alpha-deoxy alpha-amino sugar such as alpha-deoxy-alpha-fructosyllysine. In another preferred embodiment, the lysine or N-terminal amino group is carbamylated (carbamoylated), such as by virtue of reaction with cyanate ion, alkyl-carbamylated, aryl-carbamylated, or aryl-thiocarbamylated with an alkyl-isocyanate, aryl-isocyanate, or aryl-isothiocyanate, respectively, or it may be acylated by a reactive alkylcarboxylic or arylcarboxylic acid derivative, such as by reaction with acetic anhydride, succinic anhydride or phthalic anhydride. At least one lysine group or the N-terminal amino group may also be trinitrophenyl modified by reaction with a trinitrobenzenesulfonic acid, or preferably with one of its salts. In another embodiment, lysine residues may be modified by reaction with a glyoxal, such as reaction with glyoxal, methylglyoxal or 3-deoxyglucosone to form the corresponding alpha-carboxyalkyl derivatives.

[0015] In another embodiment, a tissue protective cytokine can be generated by modifying at least one tyrosine residue of erythropoietin by using an electrophilic reagent, such as but not limited to modification by nitration or iodination, to modify an aromatic ring position.

[0016] As noted above, a tissue protective agent useful for the purposes herein may have at least one of the aforementioned modifications, but may have more than one of the above modifications. By way of example of tissue protective cytokines with one modification to the amino acid portion of the molecule and optional modification to the carbohydrate portion of the molecule, a tissue protective cytokine is carbamylerythropoietin, carbamylasialoerythropoietin, carbamylhyposialoerythropoietin, acetylerythropoietin, acetylasialoerythropoietin, acetylhyposialoerythropoietin, succinylerythropoietin, succinylasialoerythropoietin, succinylhyposialoerythropoietin, biotinylerythropoietin, biotinylasialoerythropoietin, biotinylhyposialoerythropoietin, iodoerythropoietin, iodoasialoerythropoietin, iodohyposialoerythropoietin, N-epsilon-carboxymethylerythropoietin, N-epsilon-carboxymethylhyposialoerythropoietin, and glucitolylerythropoietin, glucitolylasialoerythropoietin, glucitolylasialohypoerythropoietin. These compounds are merely exemplary of the modified erythropoietins of the invention. The foregoing trivial names are merely representative of the modifications of the native erythropoietin molecule, and as hereinbefore described, the modification of the amino group may be on one or more epsilon amino groups of lysine residues, or the N-terminal amino group, or, in the instance of nitro- or iodo-modified erythropoietins, of one or more tyrosine residues. Any combination of the foregoing is embraced herein. The present invention also embraces compositions, including pharmaceutical compositions, comprising one or more of the aforementioned tissue protective cytokines. Any of such compositions may also include native erythropoietin.

[0017] In another aspect of the invention, a method is provided for the protecting, maintaining, enhancing or restoring the function or viability of responsive mammalian cells and their associated cells, tissues and organs, by administering an effective amount of any one or more of the aforementioned tissue protective cytokines. In one particular aspect of the method, the responsive mammalian cells and their associated cells, tissues or organs are distal to the vasculature by virtue of a tight endothelial cell barrier. In another particular aspect, the cells, tissues, organs or other bodily parts are isolated from a mammalian body, such as those intended for transplant or reattachment. By way of non-limiting examples, the responsive cell or tissue may be neuronal, retinal, muscle, heart, lung, liver, kidney, small intestine, adrenal cortex, adrenal medulla, capillary endothelial, testes, ovary, pancreas, skin, bones, or endometrial cells or tissue. These examples of responsive cells are merely illustrative. In a particular embodiment, the responsive cell or its associated cells, tissues, or organs are not excitable cells, tissues, or organs, or do not predominantly comprise excitable cells or tissues. In another particular embodiment, the mammalian cell, tissue or organ for which an aforementioned tissue protective cytokine may be administered are those that have expended or will expend a period of time under at least one condition adverse to the viability of the cell, tissue or organ. Such conditions may include traumatic *in-situ* hypoxia or metabolic dysfunction, surgically-induced *in-situ* hypoxia or metabolic dysfunction, or *in-situ* toxin exposure; the latter may be associated with chemotherapy or radiation therapy. In one embodiment, the invention protects against the adverse conditions resulting from cardio-pulmonary bypass.

[0018] In another aspect of the invention, any of the foregoing tissue protective cytokines can be used in the preparation of a pharmaceutical composition for *ex-vivo* treatment of cells, tissues and organs for the purpose of protecting, maintaining, enhancing, or restoring the function or viability of responsive mammalian cells and their associated cells, tissues and organs. Such *ex-vivo* treatment is useful, for example, for the preservation of cells, tissues or

organs for transplant, whether autotransplant or xenotransplant. The cells, tissue or organ may be bathed in a solution comprising tissue protective cytokines, or the perfusate instilled into the organ through the vasculature or other means, to maintain cellular functioning during the period wherein the cells, tissue or organ is not integrated with the vasculature of the donor or recipient. Administration of the perfusate may be made to a donor prior to organ harvesting, as well as to the harvested organ and to the recipient. Moreover, the aforementioned use of any tissue protective cytokine is useful whenever a cell, tissue or organ is isolated from the vasculature of the individual and thus essentially existing *ex vivo* for a period of time, the term isolated referring to restricting or clamping the vasculature of or to the cell, tissue, organ or bodily part, such as may be performed during surgery, including, in particular, cardio-pulmonary bypass surgery; bypassing the vasculature of the cell, tissue, organ or bodily part; removing the cell, tissue, organ or bodily part from the mammalian body, such may be done in advance of xenotransplantation or prior to and during autotransplantation; or traumatic amputation of a cell, tissue, organ or bodily part. Thus, this aspect of the invention pertains both to the perfusion with a tissue protective cytokine *in situ* and *ex vivo*. *Ex vivo*, the erythropoietin may be provided in a cell, tissue or organ preservation solution. For either aspect, the exposing may be by way of continuous perfusion, pulsatile perfusion, infusion, bathing, injection, or catheterization.

[0019] In yet a further aspect, the invention is directed to a method for protecting, maintaining, enhancing, or restoring the viability of a mammalian cell, tissue, organ or bodily part which includes a responsive cell or tissue, in which the cell, tissue, organ or bodily part is isolated from the mammalian body. The method includes at least exposing the isolated mammalian cell, tissue, organ or bodily part to an amount of a tissue protective cytokine as mentioned above for a duration which is effective to protect, maintain, enhance, or restore the aforesaid viability. In non-limiting examples, isolated refers to restricting or clamping the vasculature of or to the cell, tissue, organ or bodily part, such as may be performed during

surgery, in particular, cardio-pulmonary bypass surgery; bypassing the vasculature of the cell, tissue, organ or bodily part; removing the cell, tissue, organ or bodily part from the mammalian body, such may be done in advance of xenotransplantation or prior to and during autotransplantation; or traumatic amputation of a cell, tissue, organ or bodily part. Thus, this aspect of the invention pertains both to the perfusion with a tissue protective cytokine *in situ* and *ex vivo*. *Ex vivo*, the tissue protective cytokine may be provided in a cell, tissue or organ preservation solution. For either aspect, the exposing may be by way of continuous perfusion, pulsatile perfusion, infusion, bathing, injection, or catheterization.

[0020] By way of non-limiting examples, the aforementioned *ex-vivo* responsive cell or tissue may be or comprise neuronal, retinal, muscle, heart, lung, liver, kidney, small intestine, adrenal cortex, adrenal medulla, capillary endothelial, testes, ovary, pancreas, skin, bone, bone marrow, umbilical chord blood or endometrial cells or tissue. These examples of responsive cells are merely illustrative.

[0021] All of the foregoing methods and uses are preferably applicable to human beings, but are useful as well for any mammal, such as but not limited to companion animals, domesticated animals, livestock and zoo animals. Routes of administration of the aforementioned pharmaceutical compositions include oral, intravenous, intranasal, topical, intraluminal, inhalation or parenteral administration, the latter including intravenous, intraarterial, subcutaneous, intramuscular, intraperitoneal, submucosal or intradermal. For *ex-vivo* use, a perfusate or bath solution is preferred. This includes perfusing an isolated portion of the vasculature *in situ*.

[0022] In yet another aspect of the invention, any of the aforementioned tissue protective cytokines are useful in preparing a pharmaceutical composition for restoring a dysfunctional cell, tissue or organ when administered after the onset of the disease or condition responsible for the dysfunction. By way of non-limiting example, administration of a pharmaceutical composition comprising tissue protective cytokines restores cognitive function in animals previously having brain trauma, even when administered long after (*e.g.*, three days, five days, a week, a month, or longer) the trauma has subsided.

[0023] In yet another embodiment, the invention provides methods for the use of the aforementioned tissue protective cytokine for restoring a dysfunctional cell, tissue or organ when administered after the onset of the disease or condition responsible for the dysfunction. By way of non-limiting example, methods for administration of a pharmaceutical composition comprising a tissue protective cytokine restores cognitive function in animals previously having brain trauma, even when administered long after (*e.g.*, three days, five days, a week, a month, or longer) the trauma has subsided. Tissue protective cytokines useful for such methods include any of the particular aforementioned modified erythropoietins

[0024] In still yet a further aspect of the present invention, methods are provided for facilitating the transcytosis of a molecule across an endothelial cell barrier in a mammal by administration of a composition of a molecule in association with a tissue protective cytokine as described hereinabove. The association between the molecule to be transported and the tissue protective cytokine may be, for example, a labile covalent bond, a stable covalent bond, or a noncovalent association with a binding site for the molecule. Endothelial cell barriers may be the blood-brain barrier, the blood-eye barrier, the blood-testes barrier, the blood-ovary barrier and the blood-placenta barrier. Suitable molecules for transport by the method of the present invention include hormones such as growth hormone, nerve growth factor (NGF), brain-derived

neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), basic fibroblast growth factor (bFGF), transforming growth factor  $\beta 1$  (TGF $\beta 1$ ), transforming growth factor  $\beta 2$  (TGF $\beta 2$ ), transforming growth factor  $\beta 3$  (TGF $\beta 3$ ), interleukin 1, interleukin 2, interleukin 3, and interleukin 6, AZT, antibodies against tumor necrosis factor, antiviral, and immunosuppressive agents such as cyclosporin. Additionally, dyes or markers may be attached to erythropoietin or one of the tissue protective cytokines of the present invention in order to visualize cells, tissues, or organs within the brain and other barriered organs for diagnostic purposes.

[0025] It is a further aspect of the present invention to provide a composition for facilitating the transcytosis of a molecule across an endothelial cell barrier in a mammal, the composition comprising the molecule in association with a tissue protective cytokine as mentioned hereinabove. The association may be, for example, a labile covalent bond, a stable covalent bond, or a noncovalent association with a binding site for the molecule. Endothelial cell barriers may be the blood-brain barrier, the blood-eye barrier, the blood-testes barrier, the blood-ovary barrier and the blood-placenta barrier. Suitable molecules for transport by the method of the present invention include hormones such as growth hormone, nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), basic fibroblast growth factor (bFGF), transforming growth factor  $\beta 1$  (TGF $\beta 1$ ), transforming growth factor  $\beta 2$  (TGF $\beta 2$ ), transforming growth factor  $\beta 3$  (TGF $\beta 3$ ), interleukin 1, interleukin 2, interleukin 3, and interleukin 6, AZT, antibodies against tumor necrosis factor, antiviral, and immunosuppressive agents such as cyclosporin. Additionally, dyes or markers may be attached to erythropoietin or one of the tissue protective cytokines of the present invention in order to visualize cells, tissues, or organs within the brain and other barriered organs for diagnostic purposes.

[0026] In a still further aspect of the present invention, any of the aforementioned tissue protective cytokines is useful in preparing a pharmaceutical composition for facilitating the

transcytosis of a molecule across an endothelial cell barrier in a mammal. The association may be, for example, a labile covalent bond, a stable covalent bond, or a noncovalent association with a binding site for the molecule. Endothelial cell barriers may be the blood-brain barrier, the blood-eye barrier, the blood-testes barrier, the blood-ovary barrier and the blood-placenta barrier. Suitable molecules for transport by the method of the present invention include hormones, such as growth hormone, antibiotics, antivirals, dyes, markers, and anti-cancer agents, to name but a few non-limiting examples.

[0027] In one embodiment, the pharmaceutical composition of the invention comprises a therapeutically effective amount of a tissue protective cytokine, at least one anti-inflammatory agent, and a pharmaceutically acceptable carrier. In a related embodiment, the anti-inflammatory agent is selected from the group consisting of corticosteroids, glucocorticoids, steroids, non-steroidal anti-inflammatory drugs, beta-agonists, anticholinergic agents, methyl xanthines, gold injections, sulphasalazine, penicillamine, anti-angiogenic agents, dapsone, psoralens, anti-malarial agents, anti-viral agents, and antibiotics.

[0028] In one embodiment, the invention provides for a pharmaceutical composition of the invention comprises a therapeutically effective amount of a tissue protective cytokine, at least one anti-inflammatory agent and/or at least one immunomodulatory agent, and a pharmaceutically acceptable carrier, with the proviso that the anti-inflammatory agent or immunomodulatory agent is not an  $\alpha$ MSH or an anti-TNF. In a related embodiment, the anti-inflammatory agent or immunomodulatory agent is not an antibody.

[0029] In one embodiment, the invention provides for a pharmaceutical composition of the invention consists essentially of a therapeutically effective amount of a tissue protective cytokine and comprises at least one anti-inflammatory agent and/or at least one

[0030] The invention provides for a pharmaceutical composition that comprises a therapeutically effective amount of a tissue protective cytokine, at least one immunomodulatory agent, and a pharmaceutically acceptable carrier. In a related embodiment, the anti-inflammatory agent is selected from the group consisting of methothrexate, leflunomide, cyclophosphamide, cytoxan, Immuran, cyclosporine A, minocycline, azathioprine, antibiotics, methylprednisolone, corticosteroids, steroids, mycophenolate mofetil, rapamycin, mizoribine, deoxyspergualin, brequinar, malononitriloamindes, T cell receptor modulators, and cytokine receptor modulators.

[0031] The invention also provides for a pharmaceutical composition as described herein above, wherein said tissue protective cytokine is selected from the group consisting of i) an erythropoietin that lacks sialic acid moieties; ii) an erythropoietin that lacks N-linked or lacks O-linked carbohydrates; iii) an erythropoietin having a reduced carbohydrate content by treatment of native erythropoietin with at least one glycosidase; iv) an erythropoietin having at least one or more oxidized carbohydrates; v) an erythropoietin comprising at least one or more oxidized carbohydrates which is chemically reduced; vi) an erythropoietin comprising at least one or more modified arginine residues; vii) an erythropoietin comprising at least one or more modified lysine residues or a modification of the N-terminal amino group of the erythropoietin molecule; viii) an erythropoietin comprising at least a modified tyrosine residue; ix) an erythropoietin comprising at least a modified aspartic acid or a glutamic acid residue; x) an erythropoietin comprising at least a modified tryptophan residue; xi) an erythropoietin having at least one amino group removed; xii) an erythropoietin comprising at least an opening of at least one of the cystine linkages in the erythropoietin molecule; and xiii) a truncated erythropoietin.

[0032] In one embodiment, a method for treating inflammation in a mammal comprising responsive cells, tissues, and/or organs, comprises administering to a mammal a pharmaceutical composition comprising a therapeutically effective amount of a tissue protective cytokine and a pharmaceutically acceptable carrier. In a related embodiment, the tissue protective cytokine lacks at least one activity selected from the group consisting of increasing hematocrit, vasoconstriction, hyperactivating platelets, pro-coagulant activity and increasing production of thrombocytes.

[0033] In another embodiment, a method treating inflammation in a mammal comprising responsive cells, tissues, and/or organs, comprises administering to a mammal in need thereof a pharmaceutical composition comprising a prophylactically or therapeutically effective amount of a tissue protective cytokine and a pharmaceutically acceptable carrier, and administering to the mammal a prophylactically or therapeutically effective amount of one or more anti-inflammatory agents or immunomodulatory agents. In one embodiments, the anti-inflammatory agent is selected from the group consisting of a corticosteroid, a glucocorticoid, a steroid, a non-steroidal anti-inflammatory drug, a beta-agonist, a anticholinergic agent, a methyl xanthine, gold injection, a sulphasalazine, penicillamine, a anti-angiogenic agent, dapsone, psoralen, a anti-malarial agent, a anti-viral agent, and an antibiotic.

[0034] In one embodiment, the invention provides for a method treating inflammation in a mammal comprising responsive cells, tissues, and/or organs, comprises administering to a mammal in need thereof a pharmaceutical composition comprising a prophylactically or therapeutically effective amount of a tissue protective cytokine and a pharmaceutically acceptable carrier, and administering to the mammal a prophylactically or therapeutically effective amount of one or more anti-inflammatory agents or immunomodulatory agents, with the proviso that the anti-inflammatory agent or immunomodulatory agent is not an  $\alpha$ MSH or an

anti-TNF. In a related embodiment, the anti-inflammatory agent or immunomodulatory agent is not an antibody.

[0035] In another embodiment, the immunomodulatory agent is selected from the group consisting of a proteinaceous agent, a peptide mimetic, an antibody, a nucleic acid molecule, a small molecule, an organic compound, an inorganic compound, methothrexate, leflunomide, cyclophosphamide, cytoxan, Immuran, cyclosporine A, minocycline, azathioprine, an antibiotic, methylprednisolone (MP), a corticosteroid, a steroid, mycophenolate mofetil, rapamycin, mizoribine, deoxyspergualin, brequinar, a malononitriloamine, a T cell receptor modulator, and a cytokine receptor modulator.

[0036] The invention provides for a method as described herein above, wherein said tissue protective cytokine is i) an erythropoietin that lacks sialic acid moieties; ii) an erythropoietin that lacks N-linked or lacks O-linked carbohydrates; iii) an erythropoietin having a reduced carbohydrate content by treatment of native erythropoietin with at least one glycosidase; iv) an erythropoietin having at least one or more oxidized carbohydrates; v) an erythropoietin comprising at least one or more oxidized carbohydrates which is chemically reduced; vi) an erythropoietin comprising at least one or more modified arginine residues; vii) an erythropoietin comprising at least one or more modified lysine residues or a modification of the N-terminal amino group of the erythropoietin molecule; viii) an erythropoietin comprising at least a modified tyrosine residue; ix) an erythropoietin comprising at least a modified aspartic acid or a glutamic acid residue; x) an erythropoietin comprising at least a modified tryptophan residue; xi) an erythropoietin having at least one amino group removed; xii) an erythropoietin comprising at least an opening of at least one of the cystine linkages in the erythropoietin molecule; and xiii) a truncated erythropoietin.

[0037] In one embodiment, the tissue protective cytokine of the compositions and methods described herein above is asialoerythropoietin or phenylglyoxal-erythropoietin. In another embodiment, the tissue protective cytokine is capable of traversing an endothelial cell barrier. The endothelial cell barrier can be selected from the group consisting of blood-brain barrier, blood-eye barrier, blood-testis barrier, blood-ovary barrier, and blood-uterus barrier.

[0038] In one embodiment, the responsive cells, tissues, and/or organs in the mammal which are the target of the pharmaceutical compositions and methods of the invention, are selected from the group consisting of neuronal cells, muscle cells, heart, lung, liver, kidney, small intestine, adrenal cortex, adrenal medulla, capillary cells, endothelial cells, testes, ovary, endometrial cells, and stem cells. In another embodiment, the responsive mammalian cells further comprise cells selected from the group consisting of photoreceptor cells, ganglion cells, bipolar cells, horizontal cells, amacrine cells, Müller cells, myocardium cells, pace maker cells, sinoatrial node cells, sinoatrial node cells, sinus node cells, atrioventricular node cells, bundle of His cells, hepatocyte cells, stellate cells, Kupffer cells, mesangial cells, goblet cells, intestinal gland cells, enteral endocrine cells, glomerulosa cells, fasciculate cells reticularis cells, chromaffin cells, pericyte cells, Leydig cells, Sertoli cells, sperm cells, Graffian follicle cells, primordial follicle cells, endometrial stroma cells, and endometrial cells.

[0039] The invention also provides for a pharmaceutical composition and methods for treating inflammation in a mammal as described herein above, wherein said tissue protective cytokine is asialoerythropoietin. In preferred embodiments, the asialoerythropoietin is human asialoerythropoietin. The tissue protective cytokine is preferably an erythropoietin with no N-linked carbohydrates. The tissue protective cytokine is preferably an erythropoietin with no O-linked carbohydrates. In one embodiment, the tissue protective cytokine is an erythropoietin treated with at least one glycosidase. In another embodiment, the tissue protective cytokine is

periodate-oxidized erythropoietin. The periodate-oxidized erythropoietin is preferably chemically reduced with sodium cyanoborohydride. In one embodiment, the tissue protective cytokine is an erythropoietin comprising a R-glyoxal moiety on the one or more arginine residues, wherein R is aryl or alkyl moiety. The erythropoietin is preferably phenylglyoxal-erythropoietin. In another embodiment, the tissue protective cytokine is an erythropoietin in which at least one arginine residue is modified by reaction with a vicinal diketone selected from the group consisting of 2,3-butanedione and cyclohexanedione. In yet another embodiment, the tissue protective cytokine of the invention is an erythropoietin in which at least one arginine residue is reacted with 3-deoxyglucosone. In still another embodiment, the tissue protective cytokine is an erythropoietin molecule comprising at least one biotinylated lysine or N-terminal amino group. The erythropoietin molecule can be a biotinylated erythropoietin.

[0040] The invention also provides for a pharmaceutical composition and methods for treating inflammation in a mammal comprising a tissue protective cytokine that is a glucitolyl lysine erythropoietin or a fructosyl lysine erythropoietin.

[0041] In one embodiment, the tissue protective cytokine of the pharmaceutical compositions and methods of the invention is an erythropoietin having at least one carbamylated lysine residue. In another embodiment, the carbamylated erythropoietin is selected from the group consisting of alpha-N-carbamoylerythropoietin; N-epsilon-carbamoylerythropoietin; alpha-N-carbamoyl, N-epsilon-carbamoylerythropoietin; alpha-N-carbamoylasialoerythropoietin; N-epsilon-carbamoylasialoerythropoietin; alpha-N-carbamoyl, N-epsilon-carbamoylasialoerythropoietin; alpha-N-carbamoylhyposialoerythropoietin; N-epsilon-carbamoylhyposialoerythropoietin; and alpha-N-carbamoyl, N-epsilon-carbamoylhyposialoerythropoietin.

[0042] In one embodiment, the tissue protective cytokine of the pharmaceutical compositions and methods of the invention is an erythropoietin in which at least one lysine residue is acylated. In another embodiment, a lysine residue of said erythropoietin is acetylated. In yet another embodiment, the acetylated erythropoietin is selected from the group consisting of alpha-N-acetylerthropoietin; N-epsilon-acetylerthropoietin; alpha-N-acetyl, N-epsilon-acetylerthropoietin; alpha-N-acetylasialoerythropoietin; N-epsilon-acetylasialoerythropoietin; alpha-N-acetyl, N-epsilon-acetylasialoerythropoietin; alpha-N-acetylhyposialoerythropoietin; N-epsilon-acetylhyposialoerythropoietin; and alpha-N-acetyl, N-epsilon-acetylhyposialoerythropoietin.

[0043] In one embodiment, the tissue protective cytokine of the pharmaceutical compositions and methods of the invention is an erythropoietin comprising a succinylated lysine residue. In one embodiment, the erythropoietin is selected from the group consisting of alpha-N-succinylerythropoietin; N-epsilon-succinylerythropoietin; alpha-N-succinyl, N-epsilon-succinylerythropoietin; alpha-N-succinylasialoerythropoietin; N-epsilon-succinylasialoerythropoietin; alpha-N-succinyl, N-epsilon-succinylasialoerythropoietin; alpha-N-succinylhyposialoerythropoietin; N-epsilon-succinylhyposialoerythropoietin; and alpha-N-succinyl, N-epsilon-succinylhyposialoerythropoietin.

[0044] In one embodiment, the tissue protective cytokine of the pharmaceutical compositions and methods of the invention is an erythropoietin with at least one lysine residue modified by a 2, 4, 6-trinitrobenzenesulfonic acid salt. In one aspect of the invention, the salt is 2, 4, 6-trinitrobenzenesulfonate sodium.

[0045] In another embodiment, the tissue protective cytokine of the pharmaceutical compositions and methods of the invention is an erythropoietin in which at least one tyrosine residue is nitrated and/or iodinated.

[0046] In yet another embodiment, the tissue protective cytokine of the pharmaceutical compositions and methods of the invention is an erythropoietin in which an aspartic acid and/or glutamic acid residue is reacted with a carbodiimide followed by reaction with an amine. In one aspect of the invention the amine is glycynamide.

[0047] In one embodiment, the tissue protective cytokine of the pharmaceutical compositions and methods of the invention described herein above are used in treating inflammation resulting from a disease condition or trauma. In one embodiment, the trauma is selected from the group consisting of angitis, chronic bronchitis, pancreatitis, osteomyelitis, rheumatoid arthritis, glomerulonephritis, optic neuritis, temporal arteritis, encephalitis, meningitis, transverse myelitis, dermatomyositis, polymyositis, necrotizing fasciitis, hepatitis, and necrotizing enterocolitis.

[0048] In one embodiment, the tissue protective cytokine of the pharmaceutical compositions and methods of the invention inhibits inflammation resulting from cytokines produced by glial cells. In another embodiment, the inflammation is triggered by apoptosis.

[0049] According to one aspect of the invention, a tissue protective cytokine can be used for the preparation of a pharmaceutical composition for treating inflammation in a mammal comprising responsive cells, tissues, and/or organs. According to certain aspects the tissue protective cytokine lacks at least one activity selected from the group consisting of increasing hematocrit, vasoconstriction, hyperactivating platelets, pro-coagulant activity and increasing production of thrombocytes. In one embodiment, the inflammation results from a disease condition or trauma.

[0050] In another embodiment, the trauma is caused by a seizure disorder, multiple sclerosis,

stroke, hypotension, cardiac arrest, ischemia, myocardial infarction, age-related loss of cognitive function, radiation damage, cerebral palsy, neurodegenerative disease, Alzheimer's disease, Parkinson's disease, Leigh disease, AIDS dementia, memory loss, amyotrophic lateral sclerosis, alcoholism, mood disorder, anxiety disorder, attention deficit disorder, autism, Creutzfeld-Jakob disease, brain trauma, spinal cord trauma, brain ischemia, spinal cord ischemia, heart-lung bypass, chronic heart failure, macular degeneration, diabetic neuropathy, diabetic retinopathy, glaucoma, retinal ischemia, or retinal trauma.

[0051] The invention provides for the use of a tissue protective cytokine for the preparation of a pharmaceutical composition for treating inflammation in a mammal comprising responsive cells, tissues, and/or organs, wherein the pharmaceutical composition comprises a therapeutically effective amount of a tissue protective cytokine; at least one anti-inflammatory agent or immunomodulatory agent; and a pharmaceutically acceptable carrier. In one embodiment, the tissue protective cytokine is i) an erythropoietin that lacks sialic acid moieties; ii) an erythropoietin that lacks N-linked or lacks O-linked carbohydrates; iii) an erythropoietin having a reduced carbohydrate content by treatment of native erythropoietin with at least one glycosidase; iv) an erythropoietin having at least one or more oxidized carbohydrates; v) an erythropoietin comprising at least one or more oxidized carbohydrates which is chemically reduced; vi) an erythropoietin comprising at least one or more modified arginine residues; vii) an erythropoietin comprising at least one or more modified lysine residues or a modification of the N-terminal amino group of the erythropoietin molecule; viii) an erythropoietin comprising at least a modified tyrosine residue; ix) an erythropoietin comprising at least a modified aspartic acid or a glutamic acid residue; x) an erythropoietin comprising at least a modified tryptophan residue; xi) an erythropoietin having at least one amino group removed; xii) an erythropoietin comprising at least an opening of at least one of the cystine linkages in the erythropoietin molecule; and xiii) a truncated erythropoietin.

[0052] These and other aspects of the present invention will be better appreciated by reference to the following Figures and Detailed Description.

[0053] BRIEF DESCRIPTION OF THE FIGURES

[0054] **Figure 1** shows the distribution of erythropoietin receptor in normal human brain, in thin sections stained with an anti-erythropoietin antibody.

[0055] **Figure 2** is a higher power magnification of the image in Figure 1.

[0056] **Figure 3** shows, using gold-labeled secondary antibodies, the ultramicroscopic distribution of erythropoietin receptors.

[0057] **Figure 4**, prepared similarly to Figure 3, shows high density erythropoietin receptors at the luminal and anti-luminal surfaces of human brain capillaries.

[0058] **Figure 5** compares the *in-vitro* efficacy of erythropoietin and asialoerythropoietin on the viability of serum-starved P19 cells.

[0059] **Figure 6** is another experiment which compares the *in-vitro* efficacy of erythropoietin and asialoerythropoietin on the viability of serum-starved P19 cells.

[0060] **Figure 7** shows protection of erythropoietin and asialoerythropoietin in a rat focal cerebral ischemia model.

[0061] **Figure 8** shows a dose response comparing the efficacy of human erythropoietin and human asialoerythropoietin in middle cerebral artery occlusion in a model of ischemic stroke.

[0062] **Figure 9** shows the activity of iodinated erythropoietin in the P19 assay.

[0063] **Figure 10** shows the effect of biotinylated erythropoietin and asialoerythropoietin in the P19 assay.

[0064] **Figure 11** compares the *in-vitro* efficacy of erythropoietin and phenylglyoxal-modified erythropoietin on the viability of serum-starved P19 cells.

[0065] **Figure 12** shows the effect of tissue protective cytokines in the water intoxication assay.

[0066] **Figure 13** depicts the translocation of parenterally-administered erythropoietin into the cerebrospinal fluid.

[0067] **Figure 14** shows the maintenance of the function of a heart prepared for transplantation by erythropoietin.

[0068] **Figure 15** shows the protection of the myocardium from ischemic damage by erythropoietin after temporary vascular occlusion.

[0069] **Figure 16A-16D** depicts the effects of erythropoietin treatment in a rat glaucoma model.

[0070] **Figure 17** shows the extent of preservation of retinal function by erythropoietin in the rat glaucoma model.

[0071] **Figure 18** depicts the restoration of cognitive function following brain trauma by administration of erythropoietin starting five days after trauma.

[0072] **Figure 19** depicts the restoration of cognitive function following brain trauma by administration of erythropoietin starting 30 days after trauma.

[0073] **Figure 20** depicts the efficacy of human asialoerythropoietin in a kainate model of cerebral toxicity.

[0074] **Figure 21** depicts the efficacy of tissue protective cytokines in a rat spinal cord injury model.

[0075] **Figure 22** shows the efficacy of tissue protective cytokines within a rabbit spinal cord injury model.

[0076] **Figure 23A-23C** shows a coronal section of the brain cortical layer stained by hematoxylin and eosin.

[0077] **Figure 24A-24C** shows coronal sections of frontal cortex adjacent to the region of infarction stained by GFAP antibody.

[0078] **Figure 25A and 25B** shows coronal sections of brain cortical layer stained by OX-42 antibody.

[0079] **Figure 26A and 26B** shows coronal sections of brain of the rat adjacent to the region of infarction stained by OX-42 antibody.

[0080] **Figure 27** shows the efficacy of erythropoietin against inflammation in an EAE model.

[0081] **Figure 28** compares the affects of dexamethasone and erythropoietin on inflammation in the EAE model.

[0082] **Figure 29A and 29B** shows that erythropoietin suppresses inflammation associated with neuronal death.

#### [0083] DETAILED DESCRIPTION OF THE INVENTION

[0084] The methods of the invention provide for the local or systemic protection or enhancement of cells, tissues and organs within a mammalian body, under a wide variety of normal and adverse conditions, or protection of those which are destined for relocation to another mammalian body. In addition, restoration or regeneration of dysfunction is also provided. As mentioned above, the ability of an erythropoietin to cross a tight endothelial cell barrier and exert its positive effects on responsive cells (as well as other types of cells) distal to the vasculature offers the potential to prevent as well as treat a wide variety of conditions and diseases which otherwise cause significant cellular and tissue damage in an animal, including human, and moreover, permit success of heretofore unattemptable surgical procedures for which risk traditionally outweighed the benefits.

[0085] Erythropoietin is a glycoprotein hormone which in humans has a molecular weight of about 34 kDa. The mature protein comprises 165 amino acids, and the glycosyl residues

comprise about 40% of the weight of the molecule. Erythropoietin can be obtained commercially, for example, under the trademarks of PROCRIT, available from Ortho Biotech Inc., Raritan, NJ, and EPOGEN, available from Amgen, Inc., Thousand Oaks, CA. Furthermore, a variety of host systems may be used for expression and production of recombinant erythropoietin, including, but not limited to, bacteria, yeast, insect, plant, and mammalian, including human, cell systems. For example, recombinant erythropoietin produced in bacteria, which do not glycosylate or sialate the product, could be used to produce non-glycosylated forms of erythropoietin. Alternatively, recombinant erythropoietin can be produced in other systems that do glycosylate, *e.g.*, plants, including human cells. The forms of erythropoietin useful in the practice of the present invention encompass chemical modifications and/or expression-system-mediated glycosylation modifications of naturally-occurring, synthetic and recombinant forms of human and other mammalian erythropoietins.

[0086] "Responsive cell" refers to a mammalian cell whose function or viability may be maintained, promoted, enhanced, regenerated, or in any other way benefited, by exposure to an erythropoietin. Non-limiting examples of such cells include neuronal, retinal, muscle, heart, lung, liver, kidney, small intestine, adrenal cortex, adrenal medulla, capillary endothelial, testes, ovary, pancreas, skin, bone and endometrial cells. In particular, responsive cells include, without limitation, neuronal cells; retinal cells: photoreceptor (rods and cones), ganglion, bipolar, horizontal, amacrine, and Müller cells; muscle cells; heart cells: myocardium, pace maker, sinoatrial node, sinoatrial node, sinus node, and junction tissue cells (atrioventricular node and bundle of his); lung cells; liver cells: hepatocytes, stellate, and Kupffer cells; kidney cells: mesangial, renal epithelial, and tubular interstitial cells; small intestine cells: goblet, intestinal gland (crypts) and enteral endocrine cells; adrenal cortex cells: glomerulosa, fasciculate, and reticularis cells; adrenal medulla cells: chromaffin cells; capillary cells: pericyte cells; testes cells: Leydig, Sertoli, and sperm cells and their precursors; ovary cells: Graffian

follicle and primordial follicle cells; endometrial cells: endometrial stroma and endometrial cells; pancreas cell: islet of Langerhans,  $\alpha$ -cells,  $\beta$ -cells,  $\gamma$ -cells, and F-cells; skin cells; bone cells: osteoprogenitor, osteoclast and osteoblast cells; as well as the stem and endothelial cells present in the above listed organs. Moreover, such responsive cells and the benefits provided thereto by an erythropoietin may be extended to provide protection or enhancement indirectly to other cells that are not directly responsive, or of tissues or organs which contain such non-responsive cells. These other cells, or tissues or organs which benefit indirectly from the enhancement of responsive cells present as part of the cells, tissue or organ as "associated" cells, tissues and organs. Thus, benefits of an erythropoietin as described herein may be provided as a result of the presence of a small number or proportion of responsive cells in a tissue or organ, for example, excitable or neuronal tissue present in such tissue, or the Leydig cells of the testis, which makes testosterone. In one aspect, the responsive cell or its associated cells, tissues, or organs are not excitable cells, tissues, or organs, or do not predominantly comprise excitable cells or tissues.

[0087] The duration and degree of purposeful adverse conditions induced for ultimate benefit, such as high-dose chemotherapy, radiation therapy, prolonged *ex-vivo* transplant survival, and prolonged periods of surgically-induced ischemia, may be carried out by taking advantage of the invention herein. However, the invention is not so limited, but includes as one aspect, methods or compositions wherein the target responsive cells are distal to the vasculature by virtue of an endothelial-cell barrier or endothelial tight junctions. In general, the invention is directed to any responsive cells and associated cells, tissues and organs which may benefit from exposure to erythropoietin. Furthermore, cellular, tissue or organ dysfunction may be restored or regenerated after an acute adverse event (such as trauma) by exposure to erythropoietin.

[0088] The invention is directed generally to the use of erythropoietin for the preparation of pharmaceutical compositions for the aforementioned purposes in which cellular function is maintained, promoted, enhanced, regenerated, or in any other way benefited. The invention is also directed to methods for maintaining, enhancing, promoting, or regenerating cellular function by administering to a mammal an effective amount of erythropoietin as described herein. The invention is further directed to methods for maintaining, promoting, enhancing, or regenerating cellular function *ex vivo* by exposing cells, a tissue or organ to erythropoietin. The invention is also directed to a perfusate composition comprising erythropoietin for use in organ or tissue preservation.

[0089] The various methods of the invention utilize a pharmaceutical composition which at least includes erythropoietin at an effective amount for the particular route and duration of exposure to exert positive effects or benefits on responsive cells within or removed from a mammalian body. Where the target cell, tissues or organs of the intended therapy require erythropoietin to cross an endothelial cell barrier, the pharmaceutical composition includes erythropoietin at a concentration which is capable, after crossing the endothelial cell barrier, of exerting its desirable effects upon the responsive cells. Molecules capable of interacting with the erythropoietin receptor and modulating the activity of the receptor, herein referred to as erythropoietin or erythropoietin receptor activity modulators, are useful in the context of the present invention. These molecules may be, for example, naturally-occurring, synthetic, or recombinant forms of erythropoietin molecules, as described above, or other molecules which may not necessarily resemble erythropoietin in any manner, except to modulate erythropoietin responsive cell activity, as described herein.

[0090] In addition to the above identified tissue protective attributes, erythropoietin is more commonly associated with its effects on the bone marrow, *i.e.*, increased hematocrit

(erythropoiesis), vasoconstriction (high blood pressure), hyperactivation of platelets, pro-coagulant activity, and increased production of thrombocytes. However, these effects on the bone marrow may pose a risk in the chronic and acute administration of erythropoietin to treat the cellular, tissue, or organ dysfunctions discussed above. Therefore, the invention is directed generally to the use of tissue protective cytokines that consist of chemically modified erythropoietin, which preferably lack one or more of erythropoietin's effects on the bone marrow. More preferably, the tissue protective cytokines lack erythropoiesis; most preferably the tissue protective cytokines are devoid of all of erythropoietin's effects on the bone marrow. In other embodiments, the tissue protective cytokine lacks any two of the aforesaid effects, or any three of the aforesaid effects.

[0091] Furthermore, the tissue protective cytokines desirable for the uses described herein may be generated by guanidination, amidination, carbamylation (carbamylation), trinitrophenylation, acetylation, succinylation, nitration, or modification of arginine, lysine, tyrosine, tryptophan, or cysteine residues or carboxyl groups, among other procedures, such as limited proteolysis, removal of amino groups, and/or mutational substitution of arginine, lysine, tyrosine, tryptophan, or cysteine residues of erythropoietin by molecular biological techniques. Preferably, these chemical modifications affect the four recognized receptor regions – VLQRY (SEQ ID NO:1), TKVNFYAW (SEQ ID NO:2), SGIRSLTTL (SEQ ID NO:3), or SNFLRG (SEQ ID NO:4). More preferably, these receptor regions, which are basic in nature, are modified by chemical modification of the basic amino acids, arginine and lysine, within these regions. Additionally, the areas of the molecule surrounding these receptor regions may be chemically modified as well to affect the kinetics or receptor binding properties of the molecule. This produces tissue protective cytokines which maintain an adequate level of activities for specific organs and tissues but not for others, such as erythrocytes (e.g., Satake et al; 1990, *Biochim. Biophys. Acta* 1038:125-9; incorporated herein by reference in its entirety). One non-

limiting example as described hereinbelow is the modification of erythropoietin arginine residues by reaction with a glyoxal such as phenylglyoxal (according to the protocol of Takahashi, 1977, *J. Biochem.* 81:395-402). As will be seen below, such a tissue protective cytokine molecule fully retains its neurotrophic effect. Such tissue protective cytokine molecules are fully embraced for the various uses and compositions described herein.

[0092] The activity (in units) of erythropoietin and erythropoietin-like molecules is traditionally defined based on its effectiveness in stimulating red cell production in rodent models (and as derived by international standards of erythropoietin). One unit (U) of regular erythropoietin (MW of ~ 34,000) is about 8 ng of protein (1 mg protein is approximately 125,000 U). However, as the effect on erythropoiesis is incidental to the desired activities herein and may not necessarily be a detectable property of certain of the tissue protective cytokines of the invention, a definition of activity of certain tissue protective cytokines of the invention based on erythropoietic activity is inappropriate. Thus, as used herein, the activity unit of erythropoietin or the tissue protective cytokines is defined as the amount of protein required to elicit the same activity in neural or other responsive cellular systems as is elicited by WHO international standard erythropoietin in the same system. The skilled artisan will readily determine the units of a non-erythropoietic erythropoietin or related tissue protective cytokine molecule following the guidance herein.

[0093] Further to the above-mentioned tissue protective cytokines, the following discussion expands on the various tissue protective cytokines of the invention.

[0094] A tissue protective cytokine of the invention may consist of erythropoietin having at least no sialic acid moieties, referred to as asialoerythropoietin. Preferably, a tissue protective cytokine of the invention is human asialoerythropoietin. It may be prepared by desialylating

erythropoietin using a sialidase, such as is described in the manufacturer's packaging for Sialydase A from ProZyme Inc., San Leandro, California. Typically, PROZYME® GLYCOPRO® sequencing-grade SIALYDASE A™ (N-acetylneuraminase glycohydrolase, EC 3.2.1.18) is used to cleave all non-reducing terminal sialic acid residues from complex carbohydrates and glycoproteins such as erythropoietin. It will also cleave branched sialic acids (linked to an internal residue). Sialydase A is isolated from a clone of *Arthrobacter ureafaciens*.

[0095] In a non-limiting example of the foregoing procedure, erythropoietin may be subjected to desialylation by sialidase (0.025 U/mg EPO) at 37 C for 3 h, after which the erythropoietin may be desalted and concentrated. After passing over an ion exchange column using the AKTAPRIME™ system (Amersham Pharmacia Biotech), and elution with selected buffers, the fractions containing only the top two bands identified by immunoelectrophoresis (migrating at pI ~8.5 and ~7.9 on IEF gel) are selected. No significant amount of sialic acid should be detected in this preparation of the tissue protective cytokine.

[0096] In alternative embodiments, the tissue protective cytokine of the invention may be an erythropoietin having at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or 13 sialic acid residues, by partial desialylation by the aforementioned method. These tissue protective cytokines resulting from the partial desialylation of erythropoietin may also be referred to herein as hyposialoerythropoietins, and may be a homogeneous composition, with, for example, only 2 sialic acids per erythropoietin molecule, or may be a heterogeneous mixture of a variety of different degrees of sialylation, or, for example, having a low number, such as about 1 to about 4 sialic acid molecules on average per erythropoietin, or, in another example, a higher number, such as about 10 to about 13 sialic acids on average per erythropoietin molecule. Such mixtures may include asialoerythropoietin or erythropoietin.

[0097] An erythropoietin for the aforementioned uses may have at least one or more modified arginine residues. For example, the modified erythropoietin may comprise an R-glyoxal moiety on the one or more arginine residues, where R may be an aryl, heteroaryl, lower alkyl, lower alkoxy, or cycloalkyl group, or an alpha-deoxyglycitoyl group. As used herein, the term lower "alkyl" means a straight- or branched-chain saturated aliphatic hydrocarbon group preferably containing 1-6 carbon atoms. Representative of such groups are methyl, ethyl, isopropyl, isobutyl, butyl, pentyl, hexyl and the like. The term "alkoxy" means a lower alkyl group as defined above attached to the remainder of the molecule by oxygen. Examples of alkoxy include methoxy, ethoxy, propoxy, isopropoxy and the like. The term "cycloalkyl" refers to cyclic alkyl groups with from three to up to about 8 carbons, including for example cyclopropyl, cyclobutyl, cyclohexyl and the like. The term aryl refers to phenyl and naphthyl groups. The term heteroaryl refers to heterocyclic groups containing 4-10 ring members and 1-3 heteroatoms selected from the group consisting of oxygen, nitrogen and sulfur. Examples include but are not limited to isoxazolyl, phenylisoxazolyl, furyl, pyrimidinyl, quinolyl, tetrahydroquinolyl, pyridyl, imidazolyl, pyrrolidinyl, 1,2,4-triazolyl, thiazolyl, thienyl, and the like. The R group may be substituted, as for example the 2,3,4-trihydroxybutyl group of 3-deoxyglucosone. Typical examples of R-glyoxal compounds are glyoxal, methylglyoxal, 3-deoxyglucosone, and phenylglyoxal. Preferred R-glyoxal compounds are methylglyoxal or phenylglyoxal. An exemplary method for such modification may be found in Werber et al., 1975, *Isr. J. Med. Sci.* 11(11): 1169-70, using phenylglyoxal.

[0098] In a further example, at least one arginine residue may be modified by reaction with a vicinal diketone such as 2,3-butanedione or cyclohexanedione, preferably in ca. 50 millimolar borate buffer at pH 8-9. A procedure for the latter modification with 2,3-butanedione may be carried out in accordance with Riordan, 1973, *Biochemistry* 12(20): 3915-3923; and that with cyclohexanone according to Patthy et al., 1975, *J. Biol. Chem* 250(2): 565-9.

[0099] A tissue protective cytokine of the invention may comprise at least one or more modified lysine residues or a modification of the N-terminal amino group of the erythropoietin molecule, such modifications as those resulting from reaction of the lysine residue with an amino-group-modifying agent. For example, erythropoietin or aforementioned asialoerythropoietin or hyposialoerythropoietin, may be modified by acetylation, carbamylation, succinylation, oxidation and subsequent carboxymethyllysination, among other methods, to modify amino groups.

[00100] In a non-limiting example, tissue protective cytokine may be generated by carbamylating an erythropoietin, or a desialylated erythropoietin such as asialoerythropoietin, with recrystallized potassium cyanate in borate buffer, after which thorough dialysis is performed.

[00101] Likewise, an aforementioned erythropoietin may be succinylated by reaction with succinic anhydride, followed by dialysis to form a tissue protective cytokine of the present invention.

[00102] In yet another embodiment, a tissue protective cytokine may be generated by reacting erythropoietin with acetic anhydride in phosphate buffer to acetylate the erythropoietin. This reaction may be stopped by dialysis against water. The method is described in Satake et al, (1990). Chemical modification of erythropoietin: an increase in in-vitro activity by guanidination. *Biochimica et Biophysica Acta*. 1038: 125-129.

[00103] In another embodiment, the tissue protective cytokines are N<sup>ε</sup>-(carboxymethyl)lysine (CML) adducts from erythropoietin or asialoerythropoietin prepared by

reaction with glyoxylic acid and  $\text{NaBH}_3\text{CN}$  in sodium phosphate buffer, followed by dialysis.

Akhtar et al., (1999) Conformational study of  $\text{N}^\epsilon$ -(carboxymethyl)lysine adducts of recombinant  $\alpha$ -crystallins. *Current Eye Research*, 18: 270-276.

[00104] In another embodiment, a tissue protective cytokine is generated by modifying the lysine residues of erythropoietin by reaction with glyoxal derivatives, such as reaction with glyoxal, methylglyoxal and 3-deoxyglucosone to form  $\alpha$ -carboxyalkyl derivatives. Examples include reaction with glyoxal to form a carboxymethyllysine residue as in Glomb and Monnier, 1995, *J. Biol. Chem.* 270(17):10017-26, or with methylglyoxal to form a (1-carboxyethyl)lysine residue as in Degenhardt et al., 1998, *Cell. Mol. Biol. (Noisy-le-grand)* 44(7):1139-45. The modified lysine residue further may be chemically reduced. For example, the erythropoietin may be biotinylated via lysine groups, such as in accordance with the method described in Example 2, in which D-biotinoyl- $\epsilon$ -aminocaproic acid-N-hydroxysuccinimide ester was reacted with erythropoietin, followed by removal of unreacted biotin by gel filtration on a Centricon 10 column, as described by Wojchowski and Caslake, 1989, *Blood* 74(3):952-8. In this paper, the authors use three different methods of biotinylating erythropoietin, any of which may be used for the preparation of the tissue protective cytokines for the uses herein. Biotin may be added to (1) the sialic acid moieties (2) carboxylate groups or (3) amino groups.

[00105] In another preferred embodiment, the lysine may be reacted with an aldehyde or reducing sugar to form an imine, which may be stabilized by reduction as with sodium cyanoborohydride to form an N-alkylated lysine residue such as glucitolyl lysine, or which in the case of reducing sugars may be stabilized by Amadori or Heyns rearrangement to form an  $\alpha$ -deoxy  $\alpha$ -amino sugar such as  $\alpha$ -deoxy- $\alpha$ -fructosyllysine residue in the erythropoietin molecule. As an example, preparation of a fructosyllysine-modified protein by incubation with 0.5 M glucose in sodium phosphate buffer at pH 7.4 for 60 days is described by

Makita et al., 1992, J. Biol. Chem. 267:5133-5138. In another example, the lysine group may be carbamylated, such as by virtue of reaction with cyanate ion, or alkyl- or aryl-carbamylated or -thiocarbamylated with an alkyl- or aryl-isocyanate or -isothiocyanate, or it may be acylated by a reactive alkyl- or arylcarboxylic acid derivative, such as by reaction with acetic anhydride or succinic anhydride or phthalic anhydride. Exemplary are the modification of lysine groups with 4-sulfophenylisothiocyanate or with acetic anhydride, both as described in Gao et al., 1994, Proc Natl Acad Sci USA 91(25):12027-30. Lysine groups may also be trinitrophenyl modified by reaction with trinitrobenzenesulfonic acid or preferably its salts. Such methods are described below in Example 2.

[00106] At least one tyrosine residue of an erythropoietin may be modified in an aromatic ring position by an electrophilic reagent, such as by nitration or iodination to generate a tissue protective cytokine. By way of non-limiting example, erythropoietin may be reacted with tetranitromethane (Nestler et al., 1985, J. Biol. Chem. 260(12):7316-21; or iodinated as described in Example 3. For example, iodination with NaI and IODO-GEN Pre-Coated Iodination Tube (Pierce, 28601), may be carried out using erythropoietin or asialoerythropoietin in sodium phosphate buffer.

[00107] At least an aspartic acid or a glutamic acid residue of an erythropoietin may be modified, such as by reaction with a carbodiimide followed by reaction with an amine such as but not limited to glycylamide.

[00108] In another example, a tryptophan residue of an erythropoietin may be modified, such as by reaction with n-bromosuccinimide or n-chlorosuccinimide, following methods such as described in Josse et al., Chem Biol Interact 1999 May 14;119-120.

[00109] In yet another example, a tissue protective cytokine may be prepared by removing at least one amino group of a native erythropoietin, such may be achieved by reaction with ninhydrin followed by reduction of the subsequent carbonyl group by reaction with borohydride.

[00110] In still a further example, a tissue protective cytokine is provided that has at least an opening of at least one of the cystine linkages in the erythropoietin molecule by reaction with a reducing agent such as dithiothreitol, followed by reaction of the subsequent sulfhydryls with iodoacetamide, iodoacetic acid or another electrophile to prevent reformation of the disulfide linkages. As noted above, alternatively or in combination, disulfide linkages may be abolished

by altering a cysteine molecule that participates in the actual cross-link or at least one other amino acid residue that results in the inability of the erythropoietin to form at least one of the disulfide linkages present in the native molecule.

[00111] A tissue protective cytokine may be prepared by subjecting an erythropoietin to a limited chemical proteolysis that targets specific residues, for example, to cleave after tryptophan residues. Such resulting erythropoietin fragments are embraced herein.

[00112] As noted above, a tissue protective cytokine useful for the purposes herein may have at least one of the aforementioned modifications, but may have more than one of the above modifications. By way of example of a tissue protective cytokine with one modification to the carbohydrate portion of the molecule and one modification to the amino acid portion, a tissue protective cytokine may be asialoerythropoietin and have its lysine residues biotinylated or carbamylated.

[00113] Moreover, the chemically modified erythropoietin may be further modified by mutating at least one amino acid of the erythropoietin. Such mutations may include substitutions, deletions, including internal deletions, additions, including additions yielding fusion proteins, or conservative substitutions of amino acid residues within and/or adjacent to the amino acid sequence, but that result in a "silent" change, in that the change produces a functionally equivalent erythropoietin. Conservative amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues involved. For example, nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and methionine; polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine; positively charged (basic) amino acids include arginine, lysine, and histidine; and

negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Alternatively, non-conservative amino acid changes, and larger insertions and deletions may be used to create functionally altered erythropoietin. Such mutants can be used to alter erythropoietin properties in desirable ways. For example, in one embodiment, an erythropoietin useful for the practice of the invention can be altered in one or more amino acids within the four functional domains of erythropoietin which affect receptor binding: VLQRY (SEQ ID NO:1) and/or TKVNFYAW (SEQ ID NO:2) and/or SGLRSLTTL (SEQ ID NO:3) and/or SNFLRG (SEQ ID NO:4). In another embodiment, erythropoietins containing mutations in the surrounding areas of the molecule which affect the kinetics or receptor-binding properties of the molecule can be used.

[00114] These additional modifications may be used to enhance the tissue protective effect, suppress the bone marrow effect, or alter the physical properties, such as charge, of the tissue protective cytokine.

[00115] The foregoing exemplary methods for preparing tissue protective cytokines of the invention are merely illustrative and non-limiting, and these or other methods may be used to prepare the compounds of the invention. The names hereinabove wherein the method of preparation is contained within the name, such as "acetylated" or "biotinylated," are provided herein merely as a means for understanding how the compound was made, yet the present invention is directed to the compounds that are products of the aforementioned reactions. One of skill in the art would readily recognize the compounds that are the products of the reactions mentioned above. Heretofore the compounds of the invention have been referred to by informal or trivial names to convey the scope of the modifications of the invention and that they may occur at one or more sites on the erythropoietin or modified erythropoietin molecule. By ways of non-limiting examples, the following specific compounds are members of the compound groups embraced herein.

[00116] 1. Carbamylated erythropoietins: The following compounds represent carbamoyl moieties on the N-terminal amino acid of an erythropoietin molecule ("alpha-N-carbamoyl-") or on one (or more) epsilon amino groups of lysyl residues of erythropoietin ("N-epsilon-carbamoyl-"). Of course, multiple N-epsilon modifications with or without the alpha-N-modification may be present.

- i. alpha-N-carbamoylerythropoietin
- ii. N-epsilon-carbamoylerythropoietin
- iii. alpha-N-carbamoyl, N-epsilon-carbamoylerythropoietin
- iv. alpha-N-carbamoylasialoerythropoietin
- v. N-epsilon-carbamoylasialoerythropoietin
- vi. alpha-N-carbamoyl, N-epsilon-carbamoylasialoerythropoietin
- vii. alpha-N-carbamoylhyposialoerythropoietin
- viii. N-epsilon-carbamoylhyposialoerythropoietin, and
- ix. alpha-N-carbamoyl, N-epsilon-carbamoylhyposialoerythropoietin

[00117] 2. Succinylated erythropoietins: The following compounds represent succinyl moieties on the N-terminal amino acid of an erythropoietin molecule ("alpha-N-succinyl-") or on one (or more) epsilon amino groups of lysyl residues of erythropoietin ("N-epsilon-succinyl-"). Of course, multiple N-epsilon modifications with or without the alpha-N-modification may be present.

- i. alpha-N-succinylerythropoietin;
- ii. N-epsilon-succinylerythropoietin;
- iii. alpha-N-succinyl, N-epsilon-succinylerythropoietin;
- iv. alpha-N-succinylasialoerythropoietin;
- v. N-epsilon-succinylasialoerythropoietin;

- vi. alpha-N-succinyl, N-epsilon-succinylasialoerythropoietin;
- vii. alpha-N-succinylhyposialoerythropoietin;
- viii. N-epsilon-succinylhyposialoerythropoietin; and
- ix. alpha-N-succinyl, N-epsilon-succinylhyposialoerythropoietin.

[00118] 3. Acetylated erythropoietins: The following compounds represent acetyl moieties on the N-terminal amino acid of an erythropoietin molecule ("alpha-N-acetyl-") or on one (or more) epsilon amino groups of lysyl residues of erythropoietin ("N-epsilon-acetyl-"). Of course, multiple N-epsilon modifications with or without the alpha-N-modification may be present.

- i. alpha-N-acetylerthropoietin;
- ii. N-epsilon-acetylerthropoietin;
- iii. alpha-N-acetyl, N-epsilon-acetylerthropoietin;
- iv. alpha-N-acetylasialoerythropoietin;
- v. N-epsilon-acetylasialoerythropoietin;
- vi. alpha-N-acetyl, N-epsilon-acetylasialoerythropoietin;
- vii. alpha-N-acetylhyposialoerythropoietin;
- viii. N-epsilon-acetylhyposialoerythropoietin; and
- ix. alpha-N-acetyl, N-epsilon-acetylhyposialoerythropoietin.

[00119] 4. Biotinylated erythropoietins: The following compounds represent biotinyl moieties on the N-terminal amino acid of an erythropoietin molecule ("alpha-N-biotinyl-") or on one (or more) epsilon amino groups of lysyl residues of erythropoietin ("N-epsilon-biotinyl-"). Of course, multiple N-epsilon modifications with or without the alpha-N-modification may be present.

- i. alpha-N-biotinylerythropoietin;

- ii. N-epsilon-biotinylerythropoietin;
- iii. alpha-N-biotinyl, N-epsilon-biotinylerythropoietin;
- iv. alpha-N-biotinylasialoerythropoietin;
- v. N-epsilon-biotinylasialoerythropoietin;
- vi. alpha-N-biotinyl, N-epsilon-biotinylasialoerythropoietin;
- vii. alpha-N-biotinylhyposialoerythropoietin;
- viii. N-epsilon-biotinylhyposialoerythropoietin; and
- ix. alpha-N-biotinyl, N-epsilon-biotinylhyposialoerythropoietin.

[00120] 5. Iodinated erythropoietins: Of course, one of ordinary skill in the art would recognize that several different tyrosine residues as well as combinations of tyrosine residues within erythropoietin may be iodinated and that ones provided are merely illustrative.

- i. Iodoerythropoietin;
- ii. Iodoasialoerythropoietin; and
- iii. Iodohyposialoerythropoietin.

[00121] 6. Carboxymethyllysyl-erythropoietins: The following compounds represent carboxymethyl moieties on one (or more) epsilon amino groups of lysyl residues of erythropoietin ("N-epsilon-carboxymethyl-"). Of course, multiple N-epsilon modifications may be present.

- i. N-epsilon-carboxymethylerythropoietin;
- ii. N-epsilon-carboxymethylasialoerythropoietin; and
- iii. N-epsilon-carboxymethylhyposialoerythropoietin.

[00122] A variety of host-expression vector systems may be utilized to produce the erythropoietins and erythropoietin-related molecules of the invention. Such host-expression

systems represent vehicles by which the erythropoietins of interest may be produced and subsequently purified, but also represent cells that may, when transformed or transfected with the appropriate nucleotide coding sequences, exhibit the modified erythropoietin gene product *in situ*. These include but are not limited to, bacteria, insect, plant, mammalian, including human host systems, such as, but not limited to, insect cell systems infected with recombinant virus expression vectors (*e.g.*, baculovirus) containing the modified erythropoietin product coding sequences; plant cell systems infected with recombinant virus expression vectors (*e.g.*, cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (*e.g.*, Ti plasmid) containing erythropoietin-related molecule coding sequences; or mammalian cell systems, including human cell systems, (*e.g.*, HT1080, COS, CHO, BHK, 293, 3T3) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells (*e.g.*, metallothionein promoter) or from mammalian viruses (*e.g.*, the adenovirus late promoter; the vaccinia virus 7.5K promoter).

[00123] In addition, a host cell strain may be chosen that modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Such modifications (*e.g.*, glycosylation) and processing (*e.g.*, cleavage) of protein products may be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins and gene products. Appropriate cell lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells that possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product may be used. Such mammalian host cells, including human host cells, include but are not limited to HT1080, CHO, VERO, BHK, HeLa, COS, MDCK, 293, 3T3, and WI38.

[00124] For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines that stably express the erythropoietin-related molecule gene product may be engineered. Rather than using expression vectors that contain viral origins of replication, host cells can be transformed with DNA controlled by appropriate expression control elements (*e.g.*, promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of the foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci that in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines that express the erythropoietin-related molecule gene product. Such engineered cell lines may be particularly useful in screening and evaluation of compounds that affect the endogenous activity of the erythropoietin-related molecule gene product.

[00125] Alternatively, the expression characteristic of an endogenous erythropoietin gene within a cell line or microorganism may be modified by inserting a heterologous DNA regulatory element into the genome of a stable cell line or cloned microorganism such that the inserted regulatory element is operatively linked with the endogenous erythropoietin gene. For example, an endogenous erythropoietin gene which is normally "transcriptionally silent," *i.e.*, an erythropoietin gene which is normally not expressed, or is expressed only a very low levels in a cell line, may be activated by inserting a regulatory element which is capable of promoting the expression of a normally expressed gene product in that cell line or microorganism. Alternatively, a transcriptionally silent, endogenous erythropoietin gene may be activated by insertion of a promiscuous regulatory element that works across cell types.

[00126] A heterologous regulatory element may be inserted into a stable cell line or cloned microorganism, such that it is operatively linked with an endogenous erythropoietin gene, using techniques, such as targeted homologous recombination, which are well known to those of skill in the art, and described *e.g.*, in French Patent No. 2646438 to Institut Pasteur, U.S. Patent No. 4,215,051 to Chappel; U.S. Patent No. 5,578,461 to Sherwin et al.; International Application No. PCT/US92/09627 (WO93/09222) by Selden et al.; and International Application No. PCT/US90/06436 (WO91/06667) by Skoultchi *et al.*, each of which is incorporated by reference herein in its entirety.

[00127] In one embodiment of the invention, a tissue protective cytokine is a chemically modified erythropoietin molecule deficient in sialic residues, or completely lacking sialic residues, and may be produced in mammalian cell, including a human cell. Such cells may be engineered to be deficient in, or lacking, the enzymes that add sialic acids, *i.e.*, the  $\beta$ -galactoside  $\alpha$  2,3 sialyltransferase ( $\alpha$  2,3 sialyltransferase@) and the  $\beta$ -galactoside  $\alpha$  2,6 sialyltransferase ( $\alpha$  2,6 sialyltransferase@) activity. In one embodiment, a mammalian cell is used in which either or both the  $\alpha$  2,3 sialyltransferase gene and/or the  $\alpha$  2,6 sialyltransferase gene, is deleted. Such deletions may be constructed using gene knock-out techniques well known in the art. In another embodiment, dihydrofolate reductase (DHFR) deficient Chinese Hamster Ovary (CHO) cells are used as the host cell for the production of recombinant erythropoietin-related molecules. CHO cells do not express the enzyme  $\alpha$  2,6 sialyltransferase and therefore do not add sialic acid in the 2,6 linkage to N-linked oligosaccharides of glycoproteins produced in these cells. As a result, recombinant proteins produced in CHO cells lack sialic acid in the 2,6 linkage to galactose (Sasaki et al. (1987; Takeuchi et al. *supra*; Mutsaers et al Eur. J. Biochem. 156, 651 (1986); Takeuchi et al. J. Chromotgr. 400, 207 (1987). In one embodiment, to produce a host cell for the production of asialo-erythropoietin, the gene encoding  $\alpha$  2, 3 sialyltransferase in CHO cells is deleted. Such  $\alpha$  2, 3 sialyltransferase knock-out CHO cells completely lack

sialyltransferase activity, and as a result, are useful for the recombinant expression and production of a tissue protective cytokine consists of asialo-erythropoietin.

[00128] In another embodiment, asialo glycoproteins can be produced by interfering with sialic acid transport into the Golgi apparatus *e.g.*, Eckhardt et al., 1998, J. Biol. Chem. 273:20189-95). Using methods well known to those skilled in the art (*e.g.*, Oelmann et al., 2001, J. Biol. Chem. 276:26291-300), mutagenesis of the nucleotide sugar CMP-sialic acid transporter can be accomplished to produce mutants of Chinese hamster ovary cells. These cells cannot add sialic acid residues to glycoproteins such as erythropoietin and produce only asialoerythropoietin.

[00129] Transfected mammalian cells producing erythropoietin also produce cytosolic sialidase which if it leaks into the culture medium degrades sialoerythropoietin with high efficiency (*e.g.*, Gramer et al, 1995 Biotechnology 13:692-698). Using methods well known to those knowledgeable in the art (*e.g.*, from information provided in Ferrari et al, 1994, Glycobiology 4:367-373), cell lines can be transfected, mutated or otherwise caused to constitutively produce sialidase. In this manner, asialoerythropoietin can be produced during the manufacture of asialoerythropoietin.

[00130] A tissue protective cytokine of the invention has at least one modification of an amino acid residue in erythropoietin, regardless of the glycosylation state of the molecule. As mentioned above, the chemical modification may be at least a modification of at least one amino group of at least one amino acid, such as a lysine residue, or the N-terminal amino group, or iodination of at least one tyrosine residue.

[00131] Following the manufacture of the recombinant tissue protective cytokines and chemically modified recombinant tissue protective cytokines of the present invention, one of ordinary skill in the art can verify the tissue protective attributes of the cytokines and the absence of an effect on the bone marrow using well known assays.

[00132] For example, the non-erythropoietic affect of a recombinant tissue protective cytokine can be verified through the use of a TF-1 assay. In this assay TF-1 cells are grown in a complete RPMI medium supplemented with 5 ng/ml of GM-CSF and 10% FCS for a day at 37 C in a CO<sub>2</sub> incubator. The cells are then washed in and suspended at a density of 10<sup>6</sup> cells/ml for 16 hours in starvation medium (5% FCS without GM-CSF). A 96 well plate is prepared by: (1) adding 100 µl of sterile water to the outer wells to maintain moisture; (2) adding medium (10% FCS without cells or GM-CSF) alone to 5 wells; and (3) seeding 25,000 cells/well with medium containing 10% FCS and the recombinant tissue protective cytokines in remaining wells (five wells per cytokine being tested). If the cells proliferate, the recombinant tissue protective cytokine may be erythropoietic. The *in vivo* affect of the compound should then be tested on an *in vivo* assay monitoring an increase of hematocrit due to the recombinant tissue protective cytokine. A negative result – non-proliferation of cells in the TF-1 assay *in vitro* assay or no increase in hematocrit within the *in vivo* assay – means that the recombinant tissue protective cytokine is nonerythropoietic.

[00133] The tissue protective properties of the recombinant tissue protective cytokine may be verified using a P-19 *in vitro* assay or a water intoxication *in vivo* assay in rats, both of which are outlined in further detail below. The above assays are provided merely as examples, and other suitable assays to determine the effect of the cytokines on bone marrow and tissue protection are known to those of ordinary skill in the art are contemplated by the present invention as well.

[00134] In the practice of one aspect of the present invention, a pharmaceutical composition as described above containing a tissue protective cytokine may be administrable to a mammal by any route which provides a sufficient level of a tissue protective cytokine in the vasculature to permit translocation across an endothelial cell barrier and beneficial effects on responsive cells. When used for the purpose of perfusing a tissue or organ, similar results are desired. In the instance wherein the tissue protective cytokine is used for *ex-vivo* perfusion, the tissue protective cytokine may be any of the aforementioned chemically-modified erythropoietins. In the instance where the cells or tissue is non-vascularized and/or the administration is by bathing the cells or tissue with the composition of the invention, the pharmaceutical composition provides an effective responsive-cell-beneficial amount of a tissue protective cytokine. The endothelial cell barriers across which a tissue protective cytokine may translocate include tight junctions, perforated junctions, fenestrated junctions, and any other types of endothelial barriers present in a mammal. A preferred barrier is an endothelial cell tight junction, but the invention is not so limiting.

[00135] The aforementioned tissue protective cytokines are useful generally for the therapeutic or prophylactic treatment of human diseases of the central nervous system or peripheral nervous system which have primarily neurological or psychiatric symptoms, ophthalmic diseases, cardiovascular diseases, cardiopulmonary diseases, respiratory diseases, kidney, urinary and reproductive diseases, bone diseases, skin diseases, gastrointestinal diseases and endocrine and metabolic abnormalities. In particular, such conditions and diseases include hypoxic conditions, which adversely affect excitable tissues, such as excitable tissues in the central nervous system tissue, peripheral nervous system tissue, or cardiac tissue or retinal tissue such as, for example, brain, heart, or retina/eye. Therefore, the invention can be used to treat or prevent damage to excitable tissue resulting from hypoxic conditions in a variety of conditions

and circumstances. Non-limiting examples of such conditions and circumstances are provided in the table herein below.

[00136] In the example of the protection of neuronal tissue pathologies treatable in accordance with the present invention, such pathologies include those which result from reduced oxygenation of neuronal tissues. Any condition which reduces the availability of oxygen to neuronal tissue, resulting in stress, damage, and finally, neuronal cell death, can be treated by the methods of the present invention. Generally referred to as hypoxia and/or ischemia, these conditions arise from or include, but are not limited to stroke, vascular occlusion, prenatal or postnatal oxygen deprivation, suffocation, choking, near drowning, carbon monoxide poisoning, smoke inhalation, trauma, including surgery and radiotherapy, asphyxia, epilepsy, hypoglycemia, chronic obstructive pulmonary disease, emphysema, adult respiratory distress syndrome, hypotensive shock, septic shock, anaphylactic shock, insulin shock, sickle cell crisis, cardiac arrest, dysrhythmia, nitrogen narcosis, and neurological deficits caused by heart-lung bypass procedures.

[00137] In one embodiment, for example, the specific tissue protective cytokine compositions can be administered to prevent injury or tissue damage resulting from risk of injury or tissue damage during surgical procedures, such as, for example, tumor resection or aneurysm repair. Other pathologies caused by or resulting from hypoglycemia which are treatable by the methods described herein include insulin overdose, also referred to as iatrogenic hyperinsulinemia, insulinoma, growth hormone deficiency, hypocortisolism, drug overdose, and certain tumors.

[00138] Other pathologies resulting from excitable neuronal tissue damage include seizure disorders, such as epilepsy, convulsions, or chronic seizure disorders. Other treatable conditions and diseases include diseases such as stroke, multiple sclerosis, hypotension, cardiac arrest,

Alzheimer's disease, Parkinson's disease, cerebral palsy, brain or spinal cord trauma, AIDS dementia, age-related loss of cognitive function, memory loss, amyotrophic lateral sclerosis, seizure disorders, alcoholism, retinal ischemia, optic nerve damage resulting from glaucoma, and neuronal loss.

[00139] The specific composition and methods of the present invention may be used to treat inflammation resulting from disease conditions or various traumas, such as physically or chemically induced inflammation. Such traumas could include angitis, chronic bronchitis, pancreatitis, osteomyelitis, rheumatoid arthritis, glomerulonephritis, optic neuritis, temporal arteritis, encephalitis, meningitis, transverse myelitis, dermatomyositis, polymyositis, necrotizing fasciitis, hepatitis, and necrotizing enterocolitis.

[00140] Evidence has demonstrated that activated astrocytes can exert a cytotoxic role towards neurons by producing neurotoxins. Nitric oxide, reactive oxygen species, and cytokines are released from glial cells in response to cerebral ischemia (*see* Becker, K.J. 2001. Targeting the central nervous system inflammatory response in ischemic stroke. *Curr Opinion Neurol* 14:349-353 and Mattson, M.P., Culmsee, C., and Yu, Z.F. 2000. Apoptotic and Antiapoptotic mechanisms in stroke. *Cell TissueRes* 301:173-187.). Studies have further demonstrated that in models of neurodegeneration, glial activation and subsequent production of inflammatory cytokines depends upon primary neuronal damage (*see* Viviani, B., Corsini, E., Galli, C.L., Padovani, A., Ciusani, E., and Marinovich, M. 2000. Dying neural cells activate glia through the release of a protease product. *Glia* 32:84-90 and Rabuffetti, M., Scioratti, C., Tarozzo, G., Clementi, E., Manfredi, A.A., and Beltramo, M. 2000. Inhibition of caspase-1-like activity by Ac-Tyr-Val-Ala-Asp-chloromethyl ketone includes long lasting neuroprotection in cerebral ischemia through apoptosis reduction and decrease of proinflammatory cytokines. *J Neurosci* 20:4398-4404). Inflammation and glial activation is common to different forms of neuro

degenerative disorders, including cerebral ischemia, brain trauma and experimental allergic encephalomyelitis, disorders in which erythropoietin exerts a neuroprotective effect. Inhibition of cytokine production by erythropoietin could, at least in part, mediate its protective effect. However, unlike "classical" anti-inflammatory cytokines such as IL-10 and IL-13, which inhibit tumor necrosis factor production directly, erythropoietin appears to be active only in the presence of neuronal death.

[00141] While not wishing to be bound by any particular theory it appears that this anti-inflammatory activity may be hypothetically explained by several non-limiting theories. First, since erythropoietin prevents apoptosis, inflammatory events triggered by apoptosis would be prevented. Additionally, erythropoietin may prevent the release of molecular signals from dying neurons which stimulate the glia cells or could act directly on the glial cells reducing their reaction to these products. Another possibility is that erythropoietin targets more proximal members of the inflammatory cascade (*e.g.*, caspase 1, reactive oxygen or nitrogen intermediates) that trigger both apoptosis and inflammation.

[00142] Furthermore, erythropoietin appears to provide anti-inflammatory protection without the rebound affect typically associated with other anti-inflammatory compounds such as dexamethasone. Once again, not wishing to be bound by any particular theory, it appears as though this may be due to erythropoietin's affect on multipurpose neuro toxins such as nitric oxide (NO). Although activated astrocytes and microglia produce neurotoxic quantities of NO in response to various traumas, NO serves many purposes within the body including the modulation of essential physiological functions. Thus, although the use of an anti-inflammatory may alleviate inflammation by suppressing NO or other neuro toxins, if the anti-inflammatory has too long a half-life it may also interfere with these chemical's roles in repairing the damage resulting from the trauma that led to the inflammation. It is hypothesized that the tissue

protective cytokines of the present invention are able to alleviate the inflammation without interfering with the restorative capabilities of neurotoxins such as NO.

[00143] The present invention provides compositions comprising one or more tissue protective cytokines and one or more prophylactic or therapeutic agents (i.e., active agents) other than tissue protective cytokines, and methods for preventing, treating or ameliorating one or more symptoms associated with inflammation of responsive mammalian cells and their associated cells, tissues and organs in a mammal comprising administering to said mammal one or more of said compositions. The present invention also provides compositions comprising one or more tissue protective cytokines and methods for preventing, treating or ameliorating one or more symptoms associated with inflammation of responsive mammalian cells and their associated cells, tissues and organs in a mammal comprising administering to said mammal one or more of said compositions, wherein the compositions are administered in conjunction with one or more prophylactic or therapeutic agents other than tissue protective cytokines. The inflammation may be caused by injury or a disease, such as, but not limited to, disorder is asthma, encephalitis, inflammatory bowel disease, chronic obstructive pulmonary disease (COPD), arthritis, or an allergic disorder. Therapeutic or prophylactic agents include, but are not limited to, peptides, polypeptides, fusion proteins, nucleic acid molecules, small molecules, mimetic agents, synthetic drugs, inorganic molecules, and organic molecules. Any agent which is known to be useful, or which has been used or is currently being used for the prevention, treatment or amelioration of one or more symptoms associated with inflammation can be used in combination with a tissue protective cytokine in accordance with the invention described herein. Examples of such agents include, but are not limited to, dermatological agents for rashes and swellings (*e.g.*, phototherapy (*i.e.*, ultraviolet B radiation), photochemotherapy (*e.g.*, PUVA) and topical agents such as emollients, salicylic acid, coal tar, topical steroids, topical corticosteroids, topical vitamin D3 analogs (*e.g.*, calcipotriene), tazarotene, and topical

retinoids), anti-inflammatory agents (*e.g.*, corticosteroids (*e.g.*, prednisone and hydrocortisone), glucocorticoids, steroids, non-steroidal anti-inflammatory drugs (*e.g.*, aspirin, ibuprofen, diclofenac, and COX-2 inhibitors), beta-agonists, anticholinergic agents and methyl xanthines), immunomodulatory agents (*e.g.*, small organic molecules, a T cell receptor modulators, cytokine receptor modulators, T-cell depleting agents, cytokine antagonists, monokine antagonists, lymphocyte inhibitors, or anti-cancer agents), gold injections, sulphasalazine, penicillamine, anti-angiogenic agents (*e.g.*, angiostatin, TNF- $\alpha$  antagonists (*e.g.*, anti-TNF $\alpha$  antibodies), and endostatin), dapsone, psoralens (*e.g.*, methoxalen and trioxsalen), anti-malarial agents (*e.g.*, hydroxychloroquine), anti-viral agents, and antibiotics (*e.g.*, erythromycin and penicillin).

[00144] Any immunomodulatory agent well-known to one of skill in the art may be used in the methods and compositions of the invention. Immunomodulatory agents can affect one or more or all aspects of the immune response in a subject. Aspects of the immune response include, but are not limited to, the inflammatory response. In a preferred embodiment of the invention, the administration of an immunomodulatory agent to a subject inhibits or reduces one or more aspects of the subject's immune response capabilities. In a specific embodiment of the invention, the immunomodulatory agent inhibits or suppresses the inflammatory response in a subject. In one embodiment, a tissue protective cytokine of the invention is administered in combination with one or more immunomodulatory agents to treat, *i.e.* ameliorate the symptoms of, or prevent inflammation.

[00145] Examples of immunomodulatory agents include, but are not limited to, proteinaceous agents such as cytokines, peptide mimetics, and antibodies (*e.g.*, human, humanized, chimeric, monoclonal, polyclonal, Fvs, ScFvs, Fab or F(ab)<sub>2</sub> fragments or epitope binding fragments), nucleic acid molecules (*e.g.*, antisense nucleic acid molecules and triple helices), small molecules, organic compounds, and inorganic compounds. In particular, immunomodulatory

agents include, but are not limited to, methothrexate, leflunomide, cyclophosphamide, cytoxan, Immuran, cyclosporine A, minocycline, azathioprine, antibiotics (*e.g.*, FK506 (tacrolimus)), methylprednisolone (MP), corticosteroids, steroids, mycophenolate mofetil, rapamycin (sirolimus), mizoribine, deoxyspergualin, brequinar, malononitriloamindes (*e.g.*, leflunamide), T cell receptor modulators, and cytokine receptor modulators. Examples of T cell receptor modulators include, but are not limited to, anti-T cell receptor antibodies (*e.g.*, anti-CD4 antibodies (*e.g.*, cM-T412 (Boeringer), IDEC-CE9.1® (IDEC and SKB), mAB 4162W94, Orthoclone and OKTcdr4a (Janssen-Cilag)), anti-CD3 antibodies, anti-CD5 antibodies (*e.g.*, an anti-CD5 ricin-linked immunoconjugate), anti-CD7 antibodies (*e.g.*, CHH-380 (Novartis)), anti-CD8 antibodies, anti-CD40 ligand monoclonal antibodies, anti-CD52 antibodies (*e.g.*, CAMPATH 1H (Ilex)), anti-CD2 monoclonal antibodies) and CTLA4-immunoglobulin.

[00146] Examples of cytokine receptor modulators include, but are not limited to, soluble cytokine receptors (*e.g.*, the extracellular domain of a TNF- $\alpha$  receptor or a fragment thereof, the extracellular domain of an IL-1 $\beta$  receptor or a fragment thereof, and the extracellular domain of an IL-6 receptor or a fragment thereof), cytokines or fragments thereof (*e.g.*, interleukin (IL)-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-15, TNF- $\alpha$ , TNF- $\beta$ , interferon (IFN)- $\alpha$ , IFN- $\beta$ , IFN- $\gamma$ , and GM-CSF), anti-cytokine receptor antibodies (*e.g.*, anti-IL-2 receptor antibodies, anti-IL-4 receptor antibodies, anti-IL-6 receptor antibodies, anti-IL-10 receptor antibodies, and anti-IL-12 receptor antibodies), anti-cytokine antibodies (*e.g.*, anti-IFN receptor antibodies, anti-TNF- $\alpha$  antibodies, anti-IL-1 $\beta$  antibodies, anti-IL-6 antibodies, and anti-IL-12 antibodies). In a specific embodiment, a cytokine receptor modulator is IL-4, IL-10, or a fragment thereof. In another embodiment, a cytokine receptor modulator is an anti-IL-1 $\beta$  antibody, anti-IL-6 antibody, anti-IL-12 receptor antibody, anti-TNF- $\alpha$  antibody. In another embodiment, a cytokine receptor modulator is the extracellular domain of a TNF- $\alpha$  receptor or a

fragment thereof. In certain embodiments, a cytokine receptor modulator is not a TNF- $\alpha$  antagonist.

[00147] In a preferred embodiment, proteins, polypeptides or peptides (including antibodies) that are utilized as immunomodulatory agents are derived from the same species as the recipient of the proteins, polypeptides or peptides so as to reduce the likelihood of an immune response to those proteins, polypeptides or peptides. In another preferred embodiment, when the subject is a human, the proteins, polypeptides, or peptides that are utilized as immunomodulatory agents are human or humanized.

[00148] In accordance with the invention, one or more immunomodulatory agents are administered to a subject with inflammation prior to, subsequent to, or concomitantly with the therapeutic and/or prophylactic agents of the invention. Preferably, one or more immunomodulatory agents are administered to a subject with inflammation to reduce or inhibit one or more aspects of the immune response as necessary. Any technique well-known to one skilled in the art can be used to measure one or more aspects of the immune response in a particular subject, and thereby determine when it is necessary to administer an immunomodulatory agent to said subject.

[00149] In a preferred embodiment, one or more immunomodulatory agents are administered to a subject with inflammation so as to transiently reduce or inhibit one or more aspects of the immune response. Such a transient inhibition or reduction of one or more aspects of the immune system can last for hours, days, weeks, or months. Preferably, the transient inhibition or reduction in one or more aspects of the immune response last for a few hours (*e.g.*, 2 hours, 4 hours, 6 hours, 8 hours, 12 hours, 14 hours, 16 hours, 18 hours, 24 hours, 36 hours, or 48 hours), a few days (*e.g.*, 3 days, 4 days, 5 days, 6 days, 7 days, or 14 days), or a few weeks (*e.g.*, 3

weeks, 4 weeks, 5 weeks or 6 weeks). The transient reduction or inhibition of one or more aspects of the immune response enhances the prophylactic and/or therapeutic capabilities of a tissue protective cytokine.

[00150] In one embodiment of the invention, an immunomodulatory agent that reduces or depletes T cells, preferably memory T cells, is administered to a subject with inflammation in accordance with the methods of the invention. See, *e.g.*, U.S. Pat. No. 4,658,019. In another embodiment of the invention, an immunomodulatory agent that inactivates CD8<sup>+</sup> T cells is administered to a subject with inflammation in accordance with the methods of the invention. In a specific embodiment, anti-CD8 antibodies are used to reduce or deplete CD8<sup>+</sup> T cells.

[00151] The CD40 ligand (CD40L)-CD40 interaction is a desirable point to block the immune response because of its broad activity in both T helper cell activation and function as well as the absence of redundancy in its signaling pathway. Thus, in a specific embodiment of the invention, the interaction of CD40L with CD40 is transiently blocked at the time of administration of one or more of the immunomodulatory agents. This can be accomplished by treating with an agent which blocks the CD40 ligand on the TH cell and interferes with the normal binding of CD40 ligand on the T helper cell with the CD40 antigen on the B cell. An antibody to CD40 ligand (anti-CD40L) (available from Bristol-Myers Squibb Co; see, *e.g.*, European patent application 555,880, published Aug. 18, 1993) or a soluble CD40 molecule can be selected and used as an immunomodulatory agent in accordance with the methods of the invention.

[00152] Other examples of immunomodulatory agents which can be used in accordance with the invention include, but are not limited to, corticosteroids, azathioprine, mycophenolate mofetil, cyclosporin A, hydrocortisone, FK506, methotrexate, leflunomide, and

cyclophosphamide. A short course of cyclophosphamide has been demonstrated to successfully interrupt both CD4<sup>+</sup> and CD8<sup>+</sup> T cell activation to adenoviral capsid protein (Jooss et al., 1996, Hum. Gene Ther. 7:1555-1566). Hydrocortisone or cyclosporin A treatment has been successfully used to decrease the induction of cytokines, some of which may be involved in the clearance of bacterial infections and inflammation.

[00153] Nucleic acid molecules encoding proteins, polypeptides, or peptides with immunomodulatory activity or proteins, polypeptides, or peptides with immunomodulatory activity can be administered to a subject with inflammation in accordance with the methods of the invention. Further, nucleic acid molecules encoding derivatives, analogs, fragments or variants of proteins, polypeptides, or peptides with immunomodulatory activity, or derivatives, analogs, fragments or variants of proteins, polypeptides, or peptides with immunomodulatory activity can be administered to a subject with inflammation in accordance with the methods of the invention. Preferably, such derivatives, analogs, variants and fragments retain the immunomodulatory activity of the full-length wild-type protein, polypeptide, or peptide.

[00154] Proteins, polypeptides, or peptides that can be used as immunomodulatory agents can be produced by any technique well-known in the art or described herein. See, *e.g.*, Chapter 16 Ausubel et al. (eds.), 1999, Short Protocols in Molecular Biology, Fourth Edition, John Wiley & Sons, NY, which describes methods of producing proteins, polypeptides, or peptides, and which is incorporated herein by reference in its entirety. Antibodies which can be used as immunomodulatory agents can be produced by, *e.g.*, methods described in U.S. Patent No. 6,245,527 and in Harlow and Lane Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1988, which are incorporated herein by reference in their entirety. Preferably, agents that are commercially available and known to function as immunomulatory agents are used in the compositions and methods of the invention. The

immunomodulatory activity of an agent can be determined *in vitro* and/or *in vivo* by any technique well-known to one skilled in the art, including, *e.g.*, by CTL assays, proliferation assays, and immunoassays (*e.g.* ELISAs) for the expression of particular proteins such as co-stimulatory molecules and cytokines.

[00155] The specific compositions and methods of the invention may be used to treat conditions of, and damage to, retinal tissue. Such disorders include, but are not limited to retinal ischemia, macular degeneration, retinal detachment, retinitis pigmentosa, arteriosclerotic retinopathy, hypertensive retinopathy, retinal artery blockage, retinal vein blockage, hypotension, and diabetic retinopathy.

[00156] In another embodiment, the methods and principles of the invention may be used to protect or treat injury resulting from radiation damage to excitable tissue. A further utility of the methods of the present invention is in the treatment of neurotoxin poisoning, such as domoic acid shellfish poisoning, neurolathyrism, and Guam disease, amyotrophic lateral sclerosis, and Parkinson's disease.

[00157] As mentioned above, the present invention is also directed to a method for enhancing excitable tissue function in a mammal by peripheral administration of a tissue protective cytokine as described above. Various diseases and conditions are amenable to treatment using this method, and further, this method is useful for enhancing cognitive function in the absence of any condition or disease. These uses of the present invention are describe in further detail below and include enhancement of learning and training in both human and non-human mammals.

[00158] Conditions and diseases treatable by the methods of this aspect of the present invention directed to the central nervous system include but are not limited to mood disorders,

anxiety disorders, depression, autism, attention deficit hyperactivity disorder, and cognitive dysfunction. These conditions benefit from enhancement of neuronal function. Other disorders treatable in accordance with the teachings of the present invention include sleep disruption, for example, sleep apnea and travel-related disorders; subarachnoid and aneurismal bleeds, hypotensive shock, concussive injury, septic shock, anaphylactic shock, and sequelae of various encephalitides and meningitides, for example, connective tissue disease-related cerebritides such as lupus. Other uses include prevention of or protection from poisoning by neurotoxins, such as domoic acid shellfish poisoning, neurolathyrism, and Guam disease, amyotrophic lateral sclerosis, Parkinson's disease; postoperative treatment for embolic or ischemic injury; whole brain irradiation; sickle cell crisis; and eclampsia.

[00159] A further group of conditions treatable by the methods of the present invention include mitochondrial dysfunction, of either a hereditary or acquired nature, which are the cause of a variety of neurological diseases typified by neuronal injury and death. For example, Leigh disease (subacute necrotizing encephalopathy) is characterized by progressive visual loss and encephalopathy, due to neuronal drop out, and myopathy. In these cases, defective mitochondrial metabolism fails to supply enough high energy substrates to fuel the metabolism of excitable cells. An erythropoietin receptor activity modulator optimizes failing function in a variety of mitochondrial diseases. As mentioned above, hypoxic conditions adversely affect excitable tissues. The excitable tissues include, but are not limited to, central nervous system tissue, peripheral nervous system tissue, and heart tissue. In addition to the conditions described above, the methods of the present invention are useful in the treatment of inhalation poisoning such as carbon monoxide and smoke inhalation, severe asthma, adult respiratory distress syndrome, and choking and near drowning. Further conditions which create hypoxic conditions or by other means induce excitable tissue damage include hypoglycemia that may occur in inappropriate dosing of insulin, or with insulin-producing neoplasms (insulinoma).

[00160] Various neuropsychologic disorders which are believed to originate from excitable tissue damage are treatable by the instant methods. Chronic disorders in which neuronal damage is involved and for which treatment by the present invention is provided include disorders relating to the central nervous system and/or peripheral nervous system including age-related loss of cognitive function and senile dementia, chronic seizure disorders, Alzheimer's disease, Parkinson's disease, dementia, memory loss, amyotrophic lateral sclerosis, multiple sclerosis, tuberous sclerosis, Wilson's Disease, cerebral and progressive supranuclear palsy, Guam disease, Lewy body dementia, prion diseases, such as spongiform encephalopathies, e.g., Creutzfeldt-Jakob disease, Huntington's disease, myotonic dystrophy, Freidrich's ataxia and other ataxias, as well as Gilles de la Tourette's syndrome, seizure disorders such as epilepsy and chronic seizure disorder, stroke, brain or spinal cord trauma, AIDS dementia, alcoholism, autism, retinal ischemia, glaucoma, autonomic function disorders such as hypertension and sleep disorders, and neuropsychiatric disorders that include, but are not limited to schizophrenia, schizoaffective disorder, attention deficit disorder, dysthymic disorder, major depressive disorder, mania, obsessive-compulsive disorder, psychoactive substance use disorders, anxiety, panic disorder, as well as unipolar and bipolar affective disorders. Additional neuropsychiatric and neurodegenerative disorders include, for example, those listed in the American Psychiatric Association's Diagnostic and Statistical manual of Mental Disorders (DSM), the most current version of which is incorporated herein by reference in its entirety.

[00161] In another embodiment, recombinant chimeric toxin molecules comprising erythropoietin can be used for therapeutic delivery of toxins to treat a proliferative disorder, such as cancer, or viral disorder, such as subacute sclerosing panencephalitis.

[00162] The following table lists additional exemplary, non-limiting indications as to the various conditions and diseases amenable to treatment by the aforementioned tissue protective cytokines.

| <i>Cell, tissue or organ</i> | <i>Dysfunction or pathology</i> | <i>Condition or disease</i>   | <i>Type</i>   |
|------------------------------|---------------------------------|---|---|
| Heart                        | Ischemia                        | Coronary artery disease   | Acute, chronic<br>Stable, unstable  |
|                              |                                 | Myocardial infarction   | Dressler's syndrome   |
|                              |                                 | Angina  |   |
|                              |                                 | Congenital heart disease  | Valvular<br>Cardiomyopathy  |
|                              |                                 | Prinzmetal angina   |   |
|                              |                                 | Cardiac rupture   | Aneurysmatic<br>Septal perforation  |
|                              |                                 | Angiitis  |   |
|                              | Arrhythmia                      | Tachy-, bradyarrhythmia<br>Supraventricular,<br>ventricular<br>Conduction abnormalities | Stable, unstable<br>Hypersensitive carotid sinus node   |
|                              | Congestive heart failure        | Left, right, bi-ventricular   | Cardiomyopathies, such as<br>idiopathic familial, infective,<br>metabolic, storage disease,<br>deficiencies, connective tissue<br>disorder, infiltration and<br>granulomas, neurovascular |
|                              |                                 | Myocarditis   | Autoimmune, infective, idiopathic   |
|                              |                                 | Cor pulmonale   |   |
|                              | Blunt and penetrating<br>trauma |   |   |
|                              | Toxins                          | Cocaine   |   |
| Vascular                     | Hypertension                    | Primary, secondary  |   |
|                              | Decompression sickness          |   |   |
|                              | Fibromuscular<br>hyperplasia    |   |   |
|                              | Aneurysm                        | Dissecting, ruptured,<br>enlarging  |   |
| Lungs                        | Obstructive                     | Asthma<br>Chronic bronchitis,<br>Emphysema and airway<br>obstruction                    |   |
|                              | Ischemic lung disease           | Pulmonary embolism,<br>Pulmonary thrombosis,<br>Fat embolism                            |   |
|                              | Environmental lung<br>diseases  |   |   |
|                              | Ischemic lung disease           | Pulmonary embolism<br>Pulmonary thrombosis  |   |
|                              | Interstitial lung disease       | Idiopathic pulmonary<br>fibrosis  |   |
|                              | Congenital                      | Cystic fibrosis   |   |
|                              | Cor pulmonale                   |   |   |
|                              | Trauma                          |   |   |
|                              | Pneumonia and<br>pneumonitides  | Infectious, parasitic,<br>toxic, traumatic, burn,<br>aspiration                         |   |

| <i>Cell, tissue or organ</i> | <i>Dysfunction or pathology</i>  | <i>Condition or disease</i>                  | <i>Type</i>  |
|------------------------------|--|--|--|
|                              | Sarcoidosis  |  |  |
| Pancreas                     | Endocrine  | Diabetes mellitus, type I and II             | Beta cell failure, dysfunction<br>Diabetic neuropathy  |
|                              |  | Other endocrine cell failure of the pancreas |  |
|                              | Exocrine   | Exocrine pancreas failure                    | Pancreatitis   |
| Bone                         | Osteopenia   | Primary<br>secondary                         | Hypogonadism<br>immobilisation<br>Postmenopausal<br>Age-related<br>Hyperparathyroidism<br>Hyperthyroidism<br>Calcium, magnesium, phosphorus<br>and/or vitamin D deficiency |
|                              |  |  |  |
|                              |  |  |  |
|                              |  |  |  |
|                              | Osteomyelitis  |  |  |
|                              | Avascular necrosis   |  |  |
|                              | Trauma   |  |  |
|                              | Paget's disease  |  |  |
| Skin                         | Alopecia   | Areata<br>Totalis                            | Primary<br>Secondary<br>Male pattern baldness  |
|                              |  | Localized<br>Generalized                     | Primary<br>Secondary   |
|                              | Diabetic ulceration  |  |  |
|                              | Peripheral vascular disease  |  |  |
|                              | Burn injuries  |  |  |
| Autoimmune disorders         | Lupus erythematoses, Sjogren, Rheumatoid arthritis, Glomerulonephritis, Angiitis   |  |  |
|                              | Langerhan's histiocytosis  |  |  |
| Eye                          | Optic neuritis   |  |  |
|                              | Blunt and penetrating injuries, Infections, Sarcoid, Sickle C disease, Retinal detachment, Temporal arteritis  |  |  |
|                              | Retinal ischemia, macular degeneration, retinal detachment, retinitis pigmentosa, arteriosclerotic retinopathy, hypertensive retinopathy, retinal artery blockage, retinal vein blockage, hypotension, and diabetic retinopathy. |  |  |

| <i>Cell, tissue or organ</i>  | <i>Dysfunction or pathology</i>  | <i>Condition or disease</i>  | <i>Type</i>   |
|-------------------------------|--|--|---|
| Embryonic and fetal disorders | Asphyxia   |  |   |
|                               | Ischemia   |  |   |
| CNS                           | Chronic fatigue syndrome, acute and chronic hypoosmolar and hyperosmolar syndromes, AIDS Dementia, Electrocutation     |  |   |
|                               | Encephalitis   | Rabies, Herpes   |   |
|                               | Meningitis   |  |   |
|                               | Subdural hematoma  |  |   |
|                               | Nicotine addiction   |  |   |
|                               | Drug abuse and withdrawal  | Cocaine, heroin, crack, marijuana, LSD, PCP, poly-drug abuse, ecstasy, opioids, sedative hypnotics, amphetamines, caffeine |   |
|                               | Obsessive-compulsive disorders   |  |   |
|                               | Spinal stenosis, Transverse myelitis, Guillian Barre, Trauma, Nerve root compression, Tumoral compression, Heat stroke |  |   |
| ENT                           | Tinnitus<br>Meuniere's syndrome<br>Hearing loss  |  |   |
|                               | Traumatic injury, barotrauma   |  |   |
| Kidney                        | Renal failure  | Acute, chronic   | Vascular/ischemic, interstitial disease, diabetic kidney disease, nephrotic syndromes, infections |
|                               | Henoch S. Purpura  |  |   |
| Striated muscle               | Autoimmune disorders   | Myasthenia gravis<br>Dermatomyositis<br>Polymyositis   |   |
|                               | Myopathies   | Inherited metabolic, endocrine and toxic   |   |
|                               | Heat stroke  |  |   |
|                               | Crush injury   |  |   |
|                               | Rhabdomyolysis   |  |   |
|                               | Mitochondrial disease  |  |   |
|                               | Infection  | Necrotizing fasciitis  |   |
| Sexual dysfunction            | Central and peripheral   | Impotence secondary to medication  |   |
| Liver                         | Hepatitis  | Viral, bacterial, parasitic  |   |
|                               | Ischemic disease   |  |   |
|                               | Cirrhosis, fatty liver   |  |   |
|                               | Infiltrative/metabolic diseases  |  |   |
| Gastrointestinal              | Ischemic bowel disease   |  |   |

| <i>Cell, tissue or organ</i> | <i>Dysfunction or pathology</i>   | <i>Condition or disease</i>   | <i>Type</i> |
|------------------------------|-----------------------------------|---|-------------|
|                              | Inflammatory bowel disease        |   |             |
|                              | Necrotizing enterocolitis         |   |             |
| Organ transplantation        | Treatment of donor and recipient  |   |             |
| Reproductive tract           | Infertility                       | Vascular<br>Autoimmune<br>Uterine abnormalities<br>Implantation disorders |             |
| Endocrine                    | Glandular hyper- and hypofunction |   |             |

[00163] As mentioned above, these diseases, disorders or conditions are merely illustrative of the range of benefits provided by the tissue protective cytokines of the invention. Accordingly, this invention generally provides therapeutic or prophylactic treatment of the consequences of mechanical trauma or of human diseases. Therapeutic or prophylactic treatment for diseases, disorders or conditions of the CNS and/or peripheral nervous system are preferred. Therapeutic or prophylactic treatment for diseases, disorders or conditions which have a psychiatric component is provided. Therapeutic or prophylactic treatment for diseases, disorders or conditions including but not limited to those having an ophthalmic, cardiovascular, cardiopulmonary, respiratory, kidney, urinary, reproductive, gastrointestinal, endocrine, or metabolic component is provided.

[00164] One of ordinary skill in the art would understand that the pharmaceutical composition of the present invention may be made of a mixture of the tissue protective cytokines of the present invention as well as erythropoietin.

[00165] In one embodiment, such a pharmaceutical composition of erythropoietin or tissue protective cytokine may be administered systemically to protect or enhance the target cells, tissue or organ. Such administration may be parenterally, via inhalation, or transmucosally,

*e.g.*, orally, nasally, rectally, intravaginally, sublingually, submucosally or transdermally.

Preferably, administration is parenteral, *e.g.*, via intravenous or intraperitoneal injection, and also including, but is not limited to, intra-arterial, intramuscular, intradermal and subcutaneous administration.

[00166] For other routes of administration, such as by use of a perfusate, injection into an organ, or other local administration, a pharmaceutical composition will be provided which results in similar levels of a tissue protective cytokine as described above. A level of about 15pM –30 nM is preferred.

[00167] The pharmaceutical compositions of the invention may comprise a therapeutically effective amount of a compound, and a pharmaceutically acceptable carrier. In a specific embodiment, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized foreign pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is administered. Such pharmaceutical carriers can be sterile liquids, such as saline solutions in water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. A saline solution is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. These compositions can take the form of solutions, suspensions, emulsion,

tablets, pills, capsules, powders, sustained-release formulations and the like. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides. The compounds of the invention can be formulated as neutral or salt forms. Pharmaceutically acceptable salts include those formed with free amino groups such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with free carboxyl groups such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc. Examples of suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin. Such compositions will contain a therapeutically effective amount of the compound, preferably in purified form, together with a suitable amount of carrier so as to provide the form for proper administration to the patient. The formulation should suit the mode of administration.

[00168] Pharmaceutical compositions adapted for oral administration may be provided as capsules or tablets; as powders or granules; as solutions, syrups or suspensions (in aqueous or non-aqueous liquids); as edible foams or whips; or as emulsions. Tablets or hard gelatine capsules may comprise lactose, starch or derivatives thereof, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, stearic acid or salts thereof. Soft gelatine capsules may comprise vegetable oils, waxes, fats, semi-solid, or liquid polyols etc. Solutions and syrups may comprise water, polyols and sugars.

[00169] An active agent intended for oral administration may be coated with or admixed with a material that delays disintegration and/or absorption of the active agent in the gastrointestinal tract (*e.g.*, glyceryl monostearate or glyceryl distearate may be used). Thus, the sustained release of an active agent may be achieved over many hours and, if necessary, the active agent can be protected from being degraded within the stomach. Pharmaceutical compositions for oral

administration may be formulated to facilitate release of an active agent at a particular gastrointestinal location due to specific pH or enzymatic conditions.

[00170] Pharmaceutical compositions adapted for transdermal administration may be provided as discrete patches intended to remain in intimate contact with the epidermis of the recipient for a prolonged period of time. Pharmaceutical compositions adapted for topical administration may be provided as ointments, creams, suspensions, lotions, powders, solutions, pastes, gels, sprays, aerosols or oils. For topical administration to the skin, mouth, eye or other external tissues a topical ointment or cream is preferably used. When formulated in an ointment, the active ingredient may be employed with either a paraffinic or a water-miscible ointment base. Alternatively, the active ingredient may be formulated in a cream with an oil-in-water base or a water-in-oil base. Pharmaceutical compositions adapted for topical administration to the eye include eye drops. In these compositions, the active ingredient can be dissolved or suspended in a suitable carrier, *e.g.*, in an aqueous solvent. Pharmaceutical compositions adapted for topical administration in the mouth include lozenges, pastilles and mouthwashes.

[00171] Pharmaceutical compositions adapted for nasal and pulmonary administration may comprise solid carriers such as powders (preferably having a particle size in the range of 20 to 500 microns). Powders can be administered in the manner in which snuff is taken, *i.e.*, by rapid inhalation through the nose from a container of powder held close to the nose. Alternatively, compositions adopted for nasal administration may comprise liquid carriers, *e.g.*, nasal sprays or nasal drops. Alternatively, inhalation of compounds directly into the lungs may be accomplished by inhalation deeply or installation through a mouthpiece into the oropharynx. These compositions may comprise aqueous or oil solutions of the active ingredient. Compositions for administration by inhalation may be supplied in specially adapted devices including, but not limited to, pressurized aerosols, nebulizers or insufflators, which can be constructed so as to

provide predetermined dosages of the active ingredient. In a preferred embodiment, pharmaceutical compositions of the invention are administered into the nasal cavity directly or into the lungs via the nasal cavity or oropharynx.

[00172] Pharmaceutical compositions adapted for rectal administration may be provided as suppositories or enemas. Pharmaceutical compositions adapted for vaginal administration may be provided as pessaries, tampons, creams, gels, pastes, foams or spray formulations.

[00173] Pharmaceutical compositions adapted for parenteral administration include aqueous and non-aqueous sterile injectable solutions or suspensions, which may contain antioxidants, buffers, bacteriostats and solutes that render the compositions substantially isotonic with the blood of an intended recipient. Other components that may be present in such compositions include water, alcohols, polyols, glycerine and vegetable oils, for example. Compositions adapted for parenteral administration may be presented in unit-dose or multi-dose containers, for example sealed ampules and vials, and may be stored in a freeze-dried (lyophilized) condition requiring only the addition of a sterile liquid carrier, *e.g.*, sterile saline solution for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets. In one embodiment, an autoinjector comprising an injectable solution of an erythropoietin may be provided for emergency use by ambulances, emergency rooms, and battlefield situations, and even for self-administration in a domestic setting, particularly where the possibility of traumatic amputation may occur, such as by imprudent use of a lawn mower. The likelihood that cells and tissues in a severed foot or toe will survive after reattachment may be increased by administering erythropoietin or a tissue protective cytokine to multiple sites in the severed part as soon as practicable, even before the arrival of medical personnel on site, or arrival of the afflicted individual with severed toe in tow at the emergency room.

[00174] In a preferred embodiment, the composition is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the composition may also include a solubilizing agent and a local anesthetic such as lidocaine to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water-free concentrate in a hermetically-sealed container such as an ampule or sachette indicating the quantity of active agent. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by injection, an ampule of sterile saline can be provided so that the ingredients may be mixed prior to administration.

[00175] Suppositories generally contain active ingredient in the range of 0.5% to 10% by weight; oral formulations preferably contain 10% to 95% active ingredient.

[00176] A perfusate composition may be provided for use in transplanted organ baths, for in situ perfusion, or for administration to the vasculature of an organ donor prior to organ harvesting. Such pharmaceutical compositions may comprise levels of erythropoietin, tissue protective cytokines, or a form of either erythropoietin or tissue protective cytokines not suitable for acute or chronic, local or systemic administration to an individual, but will serve the functions intended herein in a cadaver, organ bath, organ perfusate, or in situ perfusate prior to removing or reducing the levels of the erythropoietin contained therein before exposing or returning the treated organ or tissue to regular circulation. The erythropoietin for this aspect of the invention may be any erythropoietin, such as naturally-occurring forms such as human erythropoietin, or any of tissue protective cytokines hereinabove described, such as asialoerythropoietin and phenylglyoxal-erythropoietins, as non-limiting examples.

[00177] The present invention provides pharmaceutical compositions for the treatment, prophylaxis, and amelioration of one or more symptoms associated with inflammation. In a specific embodiment, a composition comprises one or more tissue protective cytokines. In another embodiment, a composition comprises one or more tissue protective cytokines and one or more prophylactic or therapeutic agents other than tissue protective cytokines, said prophylactic or therapeutic agents known to be useful for, or having been or currently being used in the prevention, treatment or amelioration of one or more symptoms associated inflammation.

[00178] In a preferred embodiment, a composition of the invention is a pharmaceutical composition. Such compositions comprise a prophylactically or therapeutically effective amount of one or more prophylactic or therapeutic agents (*e.g.*, a tissue protective cytokine or other prophylactic or therapeutic agent), and a pharmaceutically acceptable carrier. In one

embodiment, the term "therapeutically effective amount" means including an amount of an agent that is not necessarily effective when the agent is administered alone but is effective when co-administered with another agent.

[00179]

[00180] The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Optionally associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

[00181] In particular, the invention provides that one or more of the prophylactic or therapeutic agents, or pharmaceutical compositions of the invention is packaged in a hermetically sealed container such as an ampoule or sachette indicating the quantity of the agent. In one embodiment, one or more of the prophylactic or therapeutic agents, or pharmaceutical compositions of the invention is supplied as a dry sterilized lyophilized powder or water free concentrate in a hermetically sealed container and can be reconstituted, *e.g.*, with water or saline to the appropriate concentration for administration to a subject. Preferably, one or more of the prophylactic or therapeutic agents, or pharmaceutical compositions of the invention is supplied as a dry sterile lyophilized powder in a hermetically sealed container at a unit dosage of at least 5 mg, more preferably at least 10 mg, at least 15 mg, at least 25 mg, at least 35 mg, at least 45 mg, at least 50 mg, at least 75 mg, or at least 100 mg. The lyophilized prophylactic or

therapeutic agents, or pharmaceutical compositions of the invention should be stored at between 2 and 8°C in its original container and the prophylactic or therapeutic agents, or pharmaceutical compositions of the invention should be administered within 1 week, preferably within 5 days, within 72 hours, within 48 hours, within 24 hours, within 12 hours, within 6 hours, within 5 hours, within 3 hours, or within 1 hour after being reconstituted. In an alternative embodiment, one or more of the prophylactic or therapeutic agents, or pharmaceutical compositions of the invention is supplied in liquid form in a hermetically sealed container indicating the quantity and concentration of the agent. Preferably, the liquid form of the administered composition is supplied in a hermetically sealed container at least 0.25 mg/ml, more preferably at least 0.5 mg/ml, at least 1 mg/ml, at least 2.5 mg/ml, at least 5 mg/ml, at least 8 mg/ml, at least 10 mg/ml, at least 15 mg/kg, at least 25 mg/ml, at least 50 mg/ml, at least 75 mg/ml or at least 100 mg/ml. The liquid form should be stored at between 2°C and 8°C in its original container.

[00182] The compositions may, if desired, be presented in a pack or dispenser device that may contain one or more unit dosage forms containing the active ingredient. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration.

[00183] Generally, the ingredients of the compositions of the invention are derived from a subject that is the same species origin or species reactivity as recipient of such compositions. Thus, in a preferred embodiment, human or humanized antibodies are administered to a human for therapy or prophylaxis.

[00184] In another embodiment, for example, a tissue protective cytokine can be delivered in a controlled-release system. For example, the polypeptide may be administered using intravenous infusion, an implantable osmotic pump, a transdermal patch, liposomes, or other

modes of administration. In one embodiment, a pump may be used (see Langer, *supra*; Sefton, 1987, CRC Crit. Ref. Biomed. Eng. 14:201; Buchwald *et al.*, 1980, Surgery 88:507; Saudek *et al.*, 1989, N. Engl. J. Med. 321:574). In another embodiment, the compound can be delivered in a vesicle, in particular a liposome (see Langer, *Science* 249:1527-1533 (1990); Treat *et al.*, in *Liposomes in the Therapy of Infectious Disease and Cancer*, Lopez-Berestein and Fidler (eds.), Liss, New York, pp. 353-365 (1989); WO 91/04014; U.S. Patent No. 4,704,355; Lopez-Berestein, *ibid.*, pp. 317-327; *see generally ibid.*). In another embodiment, polymeric materials can be used (see *Medical Applications of Controlled Release*, Langer and Wise (eds.), CRC Press: Boca Raton, Florida, 1974; *Controlled Drug Bioavailability, Drug Product Design and Performance*, Smolen and Ball (eds.), Wiley: New York (1984); Ranger and Peppas, J. Macromol. Sci. Rev. Macromol. Chem. 23:61, 1953; *see also* Levy *et al.*, 1985, *Science* 228:190; During *et al.*, 1989, *Ann. Neurol.* 25:351; Howard *et al.*, 1989, *J. Neurosurg.* 71:105).

[00185] In yet another embodiment, a controlled release system can be placed in proximity of the therapeutic target, *i.e.*, the target cells, tissue or organ, thus requiring only a fraction of the systemic dose (see, *e.g.*, Goodson, pp. 115-138 in *Medical Applications of Controlled Release*, vol. 2, *supra*, 1984). Other controlled release systems are discussed in the review by Langer (1990, *Science* 249:1527-1533).

[00186] In another embodiment, a tissue protective cytokine, as properly formulated, can be administered by nasal, oral, rectal, vaginal, or sublingual administration.

[00187] In a specific embodiment, it may be desirable to administer erythropoietin and/or the tissue protective cytokines of the invention locally to the area in need of treatment; this may be achieved by, for example, and not by way of limitation, local infusion during surgery, topical application, *e.g.*, in conjunction with a wound dressing after surgery, by injection, by means of a

catheter, by means of a suppository, or by means of an implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as silastic membranes, or fibers.

[00188] Selection of the preferred effective dose will be readily determinable by a skilled artisan based upon considering several factors, which will be known to one of ordinary skill in the art. Such factors include the particular form of erythropoietin or the tissue protective cytokine, and its pharmacokinetic parameters such as bioavailability, metabolism, half-life, etc., which will have been established during the usual development procedures typically employed in obtaining regulatory approval for a pharmaceutical compound. Further factors in considering the dose include the condition or disease to be treated or the benefit to be achieved in a normal individual, the body mass of the patient, the route of administration, whether administration is acute or chronic, concomitant medications, and other factors well known to affect the efficacy of administered pharmaceutical agents. Thus the precise dosage should be decided according to the judgment of the practitioner and each patient's circumstances, *e.g.*, depending upon the condition and the immune status of the individual patient, and according to standard clinical techniques.

[00189] In another aspect of the invention, a perfusate or perfusion solution is provided for perfusion and storage of organs for transplant, the perfusion solution includes an amount of erythropoietin or a tissue protective cytokine effective to protect responsive cells and associated cells, tissues or organs. Transplant includes but is not limited to xenotransplantation, where an organ (including cells, tissue or other bodily part) is harvested from one donor and transplanted into a different recipient; and autotransplant, where the organ is taken from one part of a body and replaced at another, including bench surgical procedures, in which an organ may be removed, and while *ex vivo*, resected, repaired, or otherwise manipulated, such as for tumor removal, and then returned to the original location. In one embodiment, the perfusion solution is

the University of Wisconsin (UW) solution (U.S. Patent No. 4,798,824) which contains from about 1 to about 25 U/ml erythropoietin, 5% hydroxyethyl starch (having a molecular weight of from about 200,000 to about 300,000 and substantially free of ethylene glycol, ethylene chlorohydrin, sodium chloride and acetone); 25mM  $\text{KH}_2\text{PO}_4$ ; 3mM glutathione; 5mM adenosine; 10mM glucose; 10mM HEPES buffer; 5mM magnesium gluconate; 1.5mM  $\text{CaCl}_2$ ; 105mM sodium gluconate; 200,000 units penicillin; 40 units insulin; 16mg dexamethasone; 12mg Phenol Red; and has a pH of 7.4-7.5 and an osmolality of about 320 mOsm/l. The solution is used to maintain cadaveric kidneys and pancreases prior to transplant. Using the solution, preservation can be extended beyond the 30-hour limit recommended for cadaveric kidney preservation. This particular perfusate is merely illustrative of a number of such solutions that can be adapted for the present use by inclusion of an effective amount of erythropoietin and/or a tissue protective cytokine. In a further embodiment, the perfusate solution contains from about 1 to about 500 ng/ml erythropoietin, or from about 40 to about 320 ng/ml erythropoietin. As mentioned above, any form of erythropoietin or tissue protective cytokines can be used in this aspect of the invention.

[00190] While the preferred recipient of a tissue protective cytokine for the purposes herein throughout is a human, the methods herein apply equally to other mammals, particularly domesticated animals, livestock, companion, and zoo animals. However, the invention is not so limiting and the benefits can be applied to any mammal.

[00191] In further aspects of the *ex-vivo* invention, erythropoietin and any tissue protective cytokine such as but not limited to the ones described above may be employed.

[00192] In another aspect of the invention, methods and compositions for enhancing the viability of cells, tissues or organs which are not isolated from the vasculature by an endothelial

cell barrier are provided by exposing the cells, tissue or organs directly to a pharmaceutical composition comprising erythropoietin or a tissue protective cytokine, or administering or contacting a pharmaceutical composition containing erythropoietin or a tissue protective cytokine to the vasculature of the tissue or organ. Enhanced activity of responsive cells in the treated tissue or organ is responsible for the positive effects exerted.

[00193] As described above, the invention is based, in part, on the discovery that erythropoietin molecules can be transported from the luminal surface to the basement membrane surface of endothelial cells of the capillaries of organs with endothelial cell tight junctions, including, for example, the brain, retina, and testis. Thus, responsive cells across the barrier are susceptible targets for the beneficial effects of erythropoietin or tissue protective cytokines, and others cell types or tissues or organs that contain and depend in whole or in part on responsive cells therein are targets for the methods of the invention. While not wishing to be bound by any particular theory, after transcytosis of erythropoietin or the tissue protective cytokine, erythropoietin or the tissue protective cytokine can interact with an erythropoietin receptor on a responsive cell, for example, neuronal, retinal, muscle, heart, lung, liver, kidney, small intestine, adrenal cortex, adrenal medulla, capillary endothelial, testes, ovary, or endometrial cell, and receptor binding can initiate a signal transduction cascade resulting in the activation of a gene expression program within the responsive cell or tissue, resulting in the protection of the cell or tissue, or organ, from damage, such as by toxins, chemotherapeutic agents, radiation therapy, hypoxia, *etc.* Thus, methods for protecting responsive cell-containing tissue from injury or hypoxic stress, and enhancing the function of such tissue are described in detail herein below.

[00194] In the practice of one embodiment of the invention, a mammalian patient is undergoing systemic chemotherapy for cancer treatment, including radiation therapy, which commonly has adverse effects such as nerve, lung, heart, ovarian or testicular damage.

Administration of a pharmaceutical composition comprising erythropoietin and/or a tissue protective cytokine as described above is performed prior to and during chemotherapy and/or radiation therapy, to protect various tissues and organs from damage by the chemotherapeutic agent, such as to protect the testes. Treatment may be continued until circulating levels of the chemotherapeutic agent have fallen below a level of potential danger to the mammalian body.

[00195] In the practice of another embodiment of the invention, various organs were planned to be harvested from a victim of an automobile accident for transplant into a number of recipients, some of which required transport for an extended distance and period of time. Prior to organ harvesting, the victim was infused with a pharmaceutical composition comprising erythropoietin and /or tissue protective cytokines as described herein. Harvested organs for shipment were perfused with a perfusate containing erythropoietin and/or tissue protective cytokines as described herein, and stored in a bath comprising erythropoietin and/or tissue protective cytokines. Certain organs were continuously perfused with a pulsatile perfusion device, utilizing a perfusate containing erythropoietin and/or tissue protective cytokines in accordance with the present invention. Minimal deterioration of organ function occurred during the transport and upon implant and reperfusion of the organs *in situ*.

[00196] In another embodiment of the invention, a surgical procedure to repair a heart valve required temporary cardioplegia and arterial occlusion. Prior to surgery, the patient was infused with a tissue protective cytokine, 4  $\mu$ g of carbamylated asialoerythropoietin per kg body weight. Such treatment prevented hypoxic ischemic cellular damage, particularly after reperfusion.

[00197] In another embodiment of the invention, in any surgical procedure, such as in cardiopulmonary bypass surgery, a naturally-occurring erythropoietin or a tissue protective cytokine of the invention can be used. In one embodiment, administration of a pharmaceutical

composition comprising erythropoietin and/or tissue protective cytokines as described above is performed prior to, during, and/or following the bypass procedure, to protect the function of brain, heart, and other organs.

[00198] In the foregoing examples in which naturally-occurring erythropoietin and/or a tissue protective cytokine of the invention is used for *ex-vivo* applications, or to treat responsive cells such as neuronal tissue, retinal tissue, heart, lung, liver, kidney, small intestine, adrenal cortex, adrenal medulla, capillary endothelial, testes, ovary, or endometrial cells or tissue, the invention provides a pharmaceutical composition in dosage unit form adapted for protection or enhancement of responsive cells, tissues or organs distal to the vasculature which comprises an amount within the range from about 1 pg to 5 mg, 500 pg to 5mg, 1 ng to 5 mg, 500 ng to 5 mg, 1  $\mu$ g to 5 mg, 500  $\mu$ g to 5 mg, or 1 mg to 5 mg of a tissue protective cytokine, and a pharmaceutically acceptable carrier. In a preferred embodiment, the amount of tissue protective cytokine is within the range from about 1 pg to 1 mg. In a preferred embodiment, the formulation contains tissue protective cytokines that are non-erythropoietic.

[00199] In a further aspect of the invention, administration of tissue protective cytokines was found to restore cognitive function in animals having undergone brain trauma. After a delay of either 5 days or 30 days, administration of erythropoietin was still able to restore function as compared to sham-treated animals, indicating the ability of an erythropoietin to regenerate or restore brain activity. Thus, the invention is also directed to the use of erythropoietin and/or tissue protective cytokines for the preparation of a pharmaceutical composition for the treatment of brain trauma and other cognitive dysfunctions, including treatment well after the injury (e.g. three days, five days, a week, a month, or longer). The invention is also directed to a method for the treatment of cognitive dysfunction following injury by administering an effective amount of

erythropoietin and/or tissue protective cytokines. Any erythropoietin and/or tissue protective cytokine as described herein may be used for this aspect of the invention.

[00200] Furthermore, this restorative aspect of the invention is directed to the use of any erythropoietins and/or tissue protective cytokines herein for the preparation of a pharmaceutical composition for the restoration of cellular, tissue or organ dysfunction, wherein treatment is initiated after, and well after, the initial insult responsible for the dysfunction. Moreover, treatment using erythropoietin and/or tissue protective cytokines of the invention can span the course of the disease or condition during the acute phase as well as a chronic phase.

[00201] In the instance wherein an erythropoietin of the invention has erythropoietic activity, in a preferred embodiment, erythropoietin may be administered systemically at a dosage between about 1  $\mu\text{g}$  and about 100  $\mu\text{g}$  /kg body weight, preferably about 5 -50  $\mu\text{g}$  /kg-body weight, most preferably about 10-30  $\mu\text{g}$  /kg-body weight, per administration. This effective dose should be sufficient to achieve serum levels of erythropoietin greater than about 10,000, 15,000, or 20,000 mU/ml (80, 120, or 160 ng/ml) of serum after erythropoietin administration. Such serum levels may be achieved at about 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 hours post-administration. Such dosages may be repeated as necessary. For example, administration may be repeated daily, as long as clinically necessary, or after an appropriate interval, *e.g.*, every 1 to 12 weeks, preferably, every 1 to 3 weeks. In one embodiment, the effective amount of erythropoietin and a pharmaceutically acceptable carrier may be packaged in a single dose vial or other container. In another embodiment, the tissue protective cytokines, which are capable of exerting the activities described herein but not causing an increase in hemoglobin concentration or hematocrit, are used. Such tissue protective cytokines are preferred in instances wherein the methods of the present invention are intended to be provided chronically. In another embodiment, an erythropoietin is given at a dose greater than that necessary to maximally

stimulate erythropoiesis. As noted above, a tissue protective cytokine of the invention does not necessarily have erythropoietic activity, and therefore the above dosages expressed in hematopoietic units are merely exemplary for erythropoietins that are erythropoietic; hereinabove weight equivalents for dosages are provided which are applicable to tissue protective cytokines.

[00202] In one embodiment, the amount of the composition of the invention which will be effective in the treatment, prevention or amelioration of one or more symptoms associated with inflammation can be determined by standard clinical techniques. The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness of the condition, and should be decided according to the judgment of the practitioner and each mammal's circumstances. Effective doses may be extrapolated from dose-response curves derived from in vitro or animal model test systems.

[00203] In a specific embodiment, the dosage of the composition of the invention or a prophylactic or therapeutic agent administered to prevent, treat or ameliorate one or more symptoms associated with an inflammatory disorder in a mammal is 150  $\mu\text{g/kg}$  or less, preferably 125  $\mu\text{g/kg}$  or less, 100  $\mu\text{g/kg}$  or less, 95  $\mu\text{g/kg}$  or less, 90  $\mu\text{g/kg}$  or less, 85  $\mu\text{g/kg}$  or less, 80  $\mu\text{g/kg}$  or less, 75  $\mu\text{g/kg}$  or less, 70  $\mu\text{g/kg}$  or less, 65  $\mu\text{g/kg}$  or less, 60  $\mu\text{g/kg}$  or less, 55  $\mu\text{g/kg}$  or less, 50  $\mu\text{g/kg}$  or less, 45  $\mu\text{g/kg}$  or less, 40  $\mu\text{g/kg}$  or less, 35  $\mu\text{g/kg}$  or less, 30  $\mu\text{g/kg}$  or less, 25  $\mu\text{g/kg}$  or less, 20  $\mu\text{g/kg}$  or less, 15  $\mu\text{g/kg}$  or less, 10  $\mu\text{g/kg}$  or less, 5  $\mu\text{g/kg}$  or less, 2.5  $\mu\text{g/kg}$  or less, 2  $\mu\text{g/kg}$  or less, 1.5  $\mu\text{g/kg}$  or less, 1  $\mu\text{g/kg}$  or less, 0.5  $\mu\text{g/kg}$  or less, or 0.5  $\mu\text{g/kg}$  or less of a mammal's body weight. In another embodiment, the dosage of the composition of the invention or a prophylactic or therapeutic agent administered to prevent, treat or ameliorate one or more symptoms associated with inflammation in a mammal is a unit dose of 0.1 mg to 20 mg, 0.1 mg to 15 mg, 0.1 mg to 12 mg, 0.1 mg to 10 mg, 0.1 mg to 8 mg, 0.1 mg to 7 mg, 0.1 mg to

5 mg, 0.1 to 2.5 mg, 0.25 mg to 20 mg, 0.25 to 15 mg, 0.25 to 12 mg, 0.25 to 10 mg, 0.25 to 8 mg, 0.25 mg to 7m g, 0.25 mg to 5 mg, 0.5 mg to 2.5 mg, 1 mg to 20 mg, 1 mg to 15 mg, 1 mg to 12 mg, 1 mg to 10 mg, 1 mg to 8 mg, 1 mg to 7 mg, 1 mg to 5 mg, or 1 mg to 2.5 mg.

[00204] In yet another embodiment, a subject is administered one or more doses of a prophylactically or therapeutically effective amount of one or more immunomodulatory agents, wherein the dose of a prophylactically or therapeutically effective amount of said agent(s) administered to said subject achieves in said subject a mean absolute lymphocyte count of approximately 500 cells/mm<sup>3</sup> to below 1500 cells/mm<sup>3</sup>, preferably below 1400 cells/mm<sup>3</sup>, below 1300 cells/mm<sup>3</sup>, below 1250 cells/mm<sup>3</sup>, below 1200 cells/mm<sup>3</sup>, below 1100 cells/mm<sup>3</sup> or below 1000 cell/mm<sup>3</sup> /mm<sup>3</sup>.

[00205] The present invention is further directed to a method for facilitating the transport of a molecule across an endothelial cell barrier in a mammal by administering a composition which comprises the particular molecule in association with an erythropoietin or tissue protective cytokine as described hereinabove. As described above, tight junctions between endothelial cells in certain organs in the body create a barrier to the entry of certain molecules. For treatment of various conditions within the barriered organ, means for facilitating passage of pharmaceutical agents is desired. Erythropoietin or tissue protective cytokines of the invention are useful as carriers for delivering other molecules across the blood-brain and other similar barriers. A composition comprising a molecule desirous of crossing the barrier with erythropoietin or a tissue protective cytokine is prepared and peripheral administration of the composition results in the transcytosis of the composition across the barrier. The association between the molecule to be transported across the barrier and the erythropoietin or tissue protective cytokine may be a labile covalent bond, in which case the molecule is released from association with the erythropoietin or tissue protective cytokine after crossing the barrier. If the

desired pharmacological activity of the molecule is maintained or unaffected by association with erythropoietin and or tissue protective cytokine, such a complex can be administered.

[00206] The skilled artisan will be aware of various means for associating molecules with erythropoietin or a tissue protective cytokine of the invention and the other agents described above, by covalent, non-covalent, and other means. Furthermore, evaluation of the efficacy of the composition can be readily determined in an experimental system. Association of molecules with erythropoietin or a tissue protective cytokine may be achieved by any number of means, including labile, covalent binding, cross-linking, etc. Biotin/avidin interactions may be employed; for example, a biotinylated erythropoietin of the invention may be complexed with a labile conjugate of avidin and a molecule desirably transported. As mentioned above, a hybrid molecule may be prepared by recombinant or synthetic means, for example, a fusion or chimeric polypeptide which includes both the domain of the molecule with desired pharmacological activity and the domain responsible for erythropoietin receptor activity modulation. Protease cleavage sites may be included in the molecule.

[00207] A molecule may be conjugated to erythropoietin or a tissue protective cytokine of the invention through a polyfunctional molecule, *i.e.*, a polyfunctional crosslinker. As used herein, the term "polyfunctional molecule" encompasses molecules having one functional group that can react more than one time in succession, such as formaldehyde, as well as molecules with more than one reactive group. As used herein, the term "reactive group" refers to a functional group on the crosslinker that reacts with a functional group on a molecule (*e.g.*, peptide, protein, carbohydrate, nucleic acid, particularly a hormone, antibiotic, or anti-cancer agent to be delivered across an endothelial cell barrier) so as to form a covalent bond between the cross-linker and that molecule. The term "functional group" retains its standard meaning in organic chemistry. The polyfunctional molecules that can be used are preferably biocompatible linkers,

*i.e.*, they are noncarcinogenic, nontoxic, and substantially non-immunogenic *in vivo*.

Polyfunctional cross-linkers such as those known in the art and described herein can be readily tested in animal models to determine their biocompatibility. The polyfunctional molecule is preferably bifunctional. As used herein, the term "bifunctional molecule" refers to a molecule with two reactive groups. The bifunctional molecule may be heterobifunctional or homobifunctional. A heterobifunctional cross-linker allows for vectorial conjugation. It is particularly preferred for the polyfunctional molecule to be sufficiently soluble in water for the cross-linking reactions to occur in aqueous solutions such as in aqueous solutions buffered at pH 6 to 8, and for the resulting conjugate to remain water soluble for more effective bio-distribution. Typically, the polyfunctional molecule covalently bonds with an amino or a sulfhydryl functional group. However, polyfunctional molecules reactive with other functional groups, such as carboxylic acids or hydroxyl groups, are contemplated in the present invention.

[00208] The homobifunctional molecules have at least two reactive functional groups, which are the same. The reactive functional groups on a homobifunctional molecule include, for example, aldehyde groups and active ester groups. Homobifunctional molecules having aldehyde groups include, for example, glutaraldehyde and subaraldehyde. The use of glutaraldehyde as a cross-linking agent was disclosed by Poznansky et al., Science 223, 1304-1306 (1984). Homobifunctional molecules having at least two active ester units include esters of dicarboxylic acids and N-hydroxysuccinimide. Some examples of such N-succinimidyl esters include disuccinimidyl suberate and dithio-bis-(succinimidyl propionate), and their soluble bis-sulfonic acid and bis-sulfonate salts such as their sodium and potassium salts. These homobifunctional reagents are available from Pierce, Rockford, Illinois.

[00209] The heterobifunctional molecules have at least two different reactive groups. The reactive groups react with different functional groups, *e.g.*, present on the erythropoietin and the

molecule. These two different functional groups that react with the reactive group on the heterobifunctional cross-linker are usually an amino group, *e.g.*, the epsilon amino group of lysine; a sulfhydryl group, *e.g.*, the thiol group of cysteine; a carboxylic acid, *e.g.*, the carboxylate on aspartic acid; or a hydroxyl group, *e.g.*, the hydroxyl group on serine.

[00210] Of course, certain of the various tissue protective cytokines of the invention, and erythropoietin, may not have suitable reactive groups available for use with certain cross-linking agent; however, one of skill in the art will be amply aware of the choice of cross-linking agents based on the available groups for cross-linking in erythropoietin or tissue protective cytokines of the invention.

[00211] When a reactive group of a heterobifunctional molecule forms a covalent bond with an amino group, the covalent bond will usually be an amido or imido bond. The reactive group that forms a covalent bond with an amino group may, for example, be an activated carboxylate group, a halocarbonyl group, or an ester group. The preferred halocarbonyl group is a chlorocarbonyl group. The ester groups are preferably reactive ester groups such as, for example, an N-hydroxy-succinimide ester group.

[00212] The other functional group typically is either a thiol group, a group capable of being converted into a thiol group, or a group that forms a covalent bond with a thiol group. The covalent bond will usually be a thioether bond or a disulfide. The reactive group that forms a covalent bond with a thiol group may, for example, be a double bond that reacts with thiol groups or an activated disulfide. A reactive group containing a double bond capable of reacting with a thiol group is the maleimido group, although others, such as acrylonitrile, are also possible. A reactive disulfide group may, for example, be a 2-pyridyldithio group or a 5, 5'-dithio-bis-(2-nitrobenzoic acid) group. Some examples of heterobifunctional reagents

containing reactive disulfide bonds include N-succinimidyl 3-(2-pyridyl-dithio) propionate (Carlsson, et al., 1978, Biochem J., 173:723-737), sodium S-4-succinimidylloxycarbonyl-alpha-methylbenzylthiosulfate, and 4-succinimidylloxycarbonyl-alpha-methyl-(2-pyridyldithio)toluene. N-succinimidyl 3-(2-pyridyldithio) propionate is preferred. Some examples of heterobifunctional reagents comprising reactive groups having a double bond that reacts with a thiol group include succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate and succinimidyl m-maleimidobenzoate.

[00213] Other heterobifunctional molecules include succinimidyl 3-(maleimido) propionate, sulfosuccinimidyl 4-(p-maleimido-phenyl) butyrate, sulfosuccinimidyl 4-(N-maleimidomethyl- cyclohexane)-1-carboxylate, maleimidobenzoyl-N-hydroxy-succinimide ester. The sodium sulfonate salt of succinimidyl m-maleimidobenzoate is preferred. Many of the above-mentioned heterobifunctional reagents and their sulfonate salts are available from Pierce Chemical Co., Rockford, Illinois USA.

[00214] The need for the above-described conjugated to be reversible or labile may be readily determined by the skilled artisan. A conjugate may be tested *in vitro* for both the erythropoietin, and for the desirable pharmacological activity. If the conjugate retains both properties, its suitability may then be tested *in vivo*. If the conjugated molecule requires separation from erythropoietin or the tissue protective cytokine for activity, a labile bond or reversible association with erythropoietin or the tissue protective cytokine will be preferable. The lability characteristics may also be tested using standard *in vitro* procedures before *in vivo* testing.

[00215] Additional information regarding how to make and use these as well as other polyfunctional reagents may be obtained from the following publications or others available in the art:

1. Carlsson, J. et al., 1978, *Biochem. J.* 173:723-737.
2. Cumber, J.A. et al., 1985, *Methods in Enzymology* 112:207-224.
3. Jue, R. et al., 1978, *Biochem* 17:5399-5405.
4. Sun, T.T. et al., 1974, *Biochem.* 13:2334-2340.
5. Blattler, W.A. et al., 1985, *Biochem.* 24:1517-152.
6. Liu, F.T. et al., 1979, *Biochem.* 18:690-697.
7. Youle, R.J. and Neville, D.M. Jr., 1980, *Proc. Natl. Acad. Sci. U.S.A.* 77:5483-5486.
8. Lerner, R.A. et al., 1981, *Proc. Natl. Acad. Sci. U.S.A.* 78:3403-3407.
9. Jung, S.M. and Moroi, M., 1983, *Biochem. Biophys. Acta* 761:162.
10. Caulfield, M.P. et al., 1984, *Biochem.* 81:7772-7776.
11. Staros, J.V., 1982, *Biochem.* 21:3950-3955.
12. Yoshitake, S. et al., 1979, *Eur. J. Biochem.* 101:395-399.
13. Yoshitake, S. et al., 1982, *J. Biochem.* 92:1413-1424.
14. Pilch, P.F. and Czech, M.P., 1979, *J. Biol. Chem.* 254:3375-3381.
15. Novick, D. et al., 1987, *J. Biol. Chem.* 262:8483-8487.
16. Lomant, A.J. and Fairbanks, G., 1976, *J. Mol. Biol.* 104:243-261.
17. Hamada, H. and Tsuruo, T., 1987, *Anal. Biochem.* 160:483-488.
18. Hashida, S. et al., 1984, *J. Applied Biochem.* 6:56-63.

[00216] Additionally, methods of cross-linking are reviewed by Means and Feeney, 1990, *Bioconjugate Chem.* 1:2-12.

[00217] Barriers which are crossed by the above-described methods and compositions of the present invention include but are not limited to the blood-brain barrier, the blood-eye barrier, the blood-testes barrier, the blood-ovary barrier, and the blood-uterus barrier.

[00218] Candidate molecules for transport across an endothelial cell barrier include, for example, hormones, such as growth hormone, neurotrophic factors, antibiotics, antivirals, or antifungals such as those normally excluded from the brain and other barriered organs, peptide radiopharmaceuticals, antisense drugs, antibodies and antivirals against biologically-active agents, pharmaceuticals, and anti-cancer agents. Non-limiting examples of such molecules include hormones such as growth hormone, nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), basic fibroblast growth factor (bFGF), transforming growth factor  $\beta 1$  (TGF $\beta 1$ ), transforming growth factor  $\beta 2$  (TGF $\beta 2$ ), transforming growth factor  $\beta 3$  (TGF $\beta 3$ ), interleukin 1, interleukin 2, interleukin 3, and interleukin 6, AZT, antibodies against tumor necrosis factor, and immunosuppressive agents such as cyclosporin. Additionally, dyes or markers may be attached to erythropoietin or one of the tissue protective cytokines of the present invention in order to visualize cells, tissues, or organs within the brain and other barriered organs for diagnostic purposes. As an example, a marker used to visualize plaque within the brain could be attached to erythropoietin or a tissue protective cytokine in order to determine the progression of Alzheimer's disease within a patient.

[00219] The present invention is also directed to a composition comprising a molecule to be transported via transcytosis across an endothelial cell tight junction barrier and an erythropoietin or tissue protective cytokine as described above. The invention is further directed to the use of a conjugate between a molecule and an erythropoietin or a tissue protective

cytokine as described above for the preparation of a pharmaceutical composition for the delivery of the molecule across a barrier as described above.

[00220] In the following examples, various animal models and in-vitro tests of neuroprotection and transcytosis are provided to demonstrate the effectiveness of the tissue protective cytokines of the invention. Such models include *in vitro* models using P-19 cells to determine the neuroprotective affects of the tissue protective cytokines, and *in-vivo* water intoxication model in mice to determine the *in vivo* neuroprotective affects of the tissue protective cytokines of the present invention. For transcytosis, model proteins conjugated to the erythropoietins of the invention are evaluated for transport into the brain following parenteral administration. These tests in in-vitro models and animal models are predictive of the efficacy of the present compounds in other mammalian species including humans. Additionally, Example 1, demonstrates that the human brain has an abundance of erythropoietin receptors that provide the mechanism for the transcytosis of erythropoietin as well as tissue protective cytokines.

[00221] The present invention may be better understood by reference to the following non-limiting Examples, which are provided as exemplary of the invention. The following examples are presented in order to more fully illustrate the preferred embodiments of the invention. They should in no way be construed, however, as limiting the broad scope of the invention.

[00222] **Example 1**

[00223] **DISTRIBUTION OF ERYTHROPOIETIN RECEPTOR  
IN HUMAN BRAIN**

[00224] Normal human brains removed during surgical procedures (e.g., to provide tumor-free margins in tumor resections) were immediately fixed in 5% acrolein in 0.1 M phosphate buffer (pH 7.4) for 3 h. Sections were cut with a vibrating microtome at 40 micrometer thickness. Immunohistochemical staining was performed using free-floating sections and the indirect antibody peroxidase-antiperoxidase method using a 1:500 dilution of erythropoietin receptor antiserum (obtained from Santa Cruz Biotechnology). Endogenous peroxidase activity was quenched by pretreatment of tissue sections with hydrogen peroxide (3% in methanol for 30 min). Tissue controls were also carried out by primary antibody omission and by using the appropriate blocking peptide (from Santa Cruz Biotech.) to confirm that staining was specific for erythropoietin (EPO) receptor.

[00225] **Figure 1** shows that capillaries of the human brain express very high levels of EPO receptor, as determined by immunohistochemistry using specific anti-EPO receptor antibodies. This provides a mechanism whereby EPO is able to penetrate into the brain from the systemic circulation, in spite of the blood brain barrier.

[00226] **Figure 2** shows the EPO receptor is densely localized within and around capillaries forming the blood brain barrier in the human brain.

[00227] A similar protocol as for **Figures 1 & 2** was performed for **Figure 3**, except that 10 micrometer sections were cut from paraffin, the embedded sections fixed by immersion in 4% paraformaldehyde. **Figure 3** shows that there is a high density of EPO receptor at the luminal and anti-luminal surfaces of human brain capillaries, forming the anatomical basis for transport of EPO from the circulation into the brain.

[00228] **Figure 4** was obtained following a similar protocol as in **Figure 3** except that the tissue was sectioned on an ultramicrotome for electron microscopy and the secondary antibody

was labeled with colloidal gold particles. This figure shows that EPO receptor is found upon the endothelial surface (\*), within cytoplasmic vesicles (arrows) and upon glial endfeet (+) in human brain, providing the anatomical basis for transport of EPO from within the circulation into the brain.

[00229]      **Example 2**

[00230]      **TISSUE PROTECTIVE CYTOKINES**

[00231]      Tissue protective cytokines desirable for the uses described herein may be generated by guanidination, carbamylation, amidination, trinitrophenylation, acetylation, succinylation, nitration, or modification of arginine or lysine residues or carboxyl groups, among other procedures as mentioned herein above, of erythropoietin. These modifications produce tissue protective cytokines that maintain their activities for specific organs and tissues but not for others, such as erythrocytes. When erythropoietin is subjected to the above reactions, it has been found that in general the resultant molecule lacks both *in-vivo* and *in-vitro* erythropoietic activity (e.g., Satake et al; 1990, *Biochim. Biophys. Acta* 1038:125-9). Some examples of the preparation of tissue protective cytokines are described below. Although the examples below use erythropoietin as the starting material, one of ordinary skill in the art would recognize that erythropoietin derivatives such as desialylated, guanidinated, carbamylated, amidinated, trinitrophenylated, acetylated, succinylated, and nitrated erythropoietin can be used as well.

[00232]      **A.      Production of Tissue Protective Cytokines by Desialylating Erythropoietin.**

[00233]      Erythropoietin may be desialylated by the following exemplary procedure. Sialidase (isolated from *Streptococcus sp* 6646K.) is obtained from SEIKAGAKU AMERICA (Code No. 120050). Erythropoietin is subjected to desialylation by sialidase (0.05 U/mg EPO)

at 37 C for 3 h. The reaction mixture is desalted and concentrated using an Ultrafree Centrifugal Filter Unit. The sample is then applied to an ion exchange column in AKTAprime™ system. The protein is eluted with selected buffers. The eluted fractions containing a significant amount of protein are then subjected to IEF gel analysis. The fractions containing only the top two bands (migrating at pI ~8.5 and ~7.9 on IEF gel) are pooled. The protein content of the pooled fractions was determined and 1/9 volumes of 10 x salt solution (1 M NaCl, 0.2 M sodium citrate, 3 mM citric acid) was added. The sialic acid content of the solution was then determined. No significant sialic content should be detected.

[00234] Asialoerythropoietin and phenylglyoxalerythropoietin were as effective as native erythropoietin for neural cells *in vitro* as shown in Figures 5-6. *In-vitro* testing was carried out using neural-like embryonal carcinoma cells (P19) that undergo apoptosis upon the withdrawal of serum. Twenty-four hours before the removal of serum, 1-1000 ng/ml of erythropoietin or a modified erythropoietin was added to the cultures. The following day the medium was removed, the cells washed with fresh, non-serum containing medium, and medium containing the test substance (no serum) added back to the cultures for an additional 48 hours. To determine the number of viable cells, a tetrazolium reduction assay was performed (CellTiter 96; Promega, Inc.). As Figure 5-6 illustrate, asialoerythropoietin appears to be of equal potency to erythropoietin itself in preventing cell death.

[00235] Retention of neuroprotective activity *in vivo* was confirmed using a rat focal ischemia model in which a reversible lesion in the territory of the middle cerebral artery is performed as described previously (Brines et al., 2000, *Proc. Nat. Acad. Sci. U.S.A.* 97:10526-31). Adult male Sprague-Dawley rats were administered asialoerythropoietin or erythropoietin (5000 U (40 µg)/kgBW intraperitoneally) or vehicle at the onset of the arterial occlusion. Twenty-four hours later, the animals were sacrificed and their brains removed for study. Serial sections were cut and stained with tetrazolium salts to identify living regions of the brain. As

shown in **Figure 7**, asialoerythropoietin was as effective as native erythropoietin in providing neuroprotection from 1 hour of ischemia. **Figure 8** shows the results of another focal ischemia model in which a comparative dose response was performed with erythropoietin and asialoerythropoietin. At the lowest dose of 250 U (2 $\mu$ g)/kg, asialoerythropoietin afforded protection whereas unmodified erythropoietin did not.

[00236]      **B.      Preparation of Tissue Protective Cytokines by Carbamylating Erythropoietin.**

[00237]      Native erythropoietin may be used to prepare the respective carbamylated molecules, in accordance with the following procedure, as described in Jin Zeng (1991). Lysine modification of metallothionein by carbamylation and guanidination. *Methods in Enzymology* 205: 433-437. First, potassium cyanate was recrystallized. A 1 M Borate buffer (pH 8.8) was prepared. An erythropoietin solution was mixed with an equal volume of the borate buffer. Potassium cyanate was added directly to the reaction tube to a final concentration of 0.5 M. The solution was mixed well and incubated at 37 C for 6 h. The solution was then dialyzed thoroughly using distilled water. The product was removed from the dialysis tubing and collected into a fresh tube. The volume was measured and 1/9 volume of 10 X salt solution (1 M NaCl, 0.2 M sodium citrate, 3 mM citric acid) is added to the solution. The protein content is determined and the product recovery rate is calculated. The products were analyzed by IEF gel followed by an *in vitro* test with TF-1 cells.

[00238]      **C.      Preparation of Tissue Protective Cytokines by Succinylating Erythropoietin.**

[00239]      Native erythropoietin may be used to prepare the respective succinylated molecules, in accordance with the following procedure, as described in Alcalde et al. (2001). Succinylation of cyclodextrin glycosyltransferase from *Thermoanaerobacter* sp. 501 enhances its transferase activity using starch as donor. *J. Biotechnology* 86: 71-80. Erythropoietin (100 ug) in 0.5 M NaHCO<sub>3</sub> (pH 8.0) was incubated with a 15 molar excess of succinic anhydride at 15 C for 1 hour. The reaction was stopped by dialysis against distilled water.

[00240]      Another method for succinylating erythropoietin is to dissolve succinic anhydride in dry acetone at 27 mg/ml. The reaction is performed in an eppendorf tube in 10 mM sodium phosphate buffer (pH 8.0). Erythropoietin and 50-fold molar of succinic anhydride are added to the tube. The solution is mixed well and the tube is rotated at 4 C for 1 h. The reaction is stopped by dialysis against 10 mM sodium phosphate buffer, using a Dialysis cassette (Slide-A-Laze 7K, Pierce 66373). The product is removed from the dialysis cassette and collected into a fresh tube. The volume is measured and 1/9 volume of 10 X salt solution (1 M NaCl, 0.2 M sodium citrate, 3 mM citric acid) is added. Determine the protein content and calculate the product recovery rate. The products were analyzed by IEF gel followed by an *in vitro* test with TF-1 cells.

[00241]      **D.      Preparation Tissue Protective Cytokine by Acetylating Erythropoietin.**

[00242]      Native erythropoietin may be used to prepare the respective acetylated molecules, in accordance with the following procedure, as described in Satake et al (1990). Chemical

modification of erythropoietin: an increase in *in-vitro* activity by guanidination. *Biochimica et Biophysica Acta*. 1038: 125-129.

[00243] The reaction was performed in an eppendorf tube in 80 mM sodium phosphate buffer (pH 7.2). Erythropoietin and equal molar of acetic anhydride were added to the tube. After mixing well, the solution was incubated on ice for 1 h. The reaction was stopped by dialysis against water, using a Dialysis cassette (Slide-A-Laze 7K, Pierce 66373). The product was removed from the dialysis cassette and collected into a fresh tube. After measuring the volume of product, 1/9 volume of 10 X salt solution (1 M NaCl, 0.2 M sodium citrate, 3 mM citric acid) was added. The protein content is determined, and the product recovery rate was calculated. The product was analyzed by IEF gel followed by an *in vitro* test with TF-1 cells.

[00244] **E. Preparation of Tissue Protective Cytokine by Carboxymethylating Lysine of Erythropoietin.**

[00245] Native erythropoietin may be used to prepare the respective N<sup>ε</sup>-(carboxymethyl)lysine (CML) modified molecules in which one or more lysyl residues of the erythropoietin are modified, in accordance with the following procedure, as described in Akhtar et al (1999) Conformational study of N<sup>ε</sup>-(carboxymethyl)lysine adducts of recombinant  $\alpha$ -crystallins. *Current Eye Research*, 18: 270-276.

[00246] Glyoxylic acid (200 mM) and NaBH<sub>3</sub>CN (120 mM) were prepared in sodium phosphate buffer (50 mM, pH 7.5). In an eppendorf tube, erythropoietin was added (in phosphate buffer). The lysine equivalent in the solution (about 8 lysine residues/mol) was then calculated. Next, 3-times greater NaBH<sub>3</sub>CN and 5 or 10-times greater glyoxylic acid was added to the tube. Each tube was vortexed and incubated at 37 C for 5 h. The samples were dialated against phosphate buffer overnight at 4 C. The volume of each product was measured after

dialysis. The protein concentration was determined, and the product recovery rate was calculated. The product was analyzed by IEF gel followed by an *in vitro* test with TF-1 cells.

[00247]      **F.      Preparation of Tissue Protective Cytokine by Iodinating Erythropoietin**

[00248]      Native erythropoietin may be used to prepare the respective iodinated molecules, in accordance with the following procedure, as described in instruction provided by Pierce Chemical Company (Rockford, IL) for ODO-Gen Pre-Coated Iodination Tubes (product # 28601).

[00249]      First, 0.1 M of NaI was prepared, and iodination was performed in an IODO-Gen Pre-Coated Iodination Tube (Pierce, 28601), with a total reaction volume of 0.1 ml/tube in sodium phosphate buffer (40 mM, pH 7.4). The protein substrate (erythropoietin) was mixed with sodium phosphate buffer and then transferred to an IODO-Gen Pre-Coated Iodination Tube. NaI was added to a final concentration of 1 - 2 mM, making the molar ration of NaI/protein as 14-20. The solution was then mixed well and incubated at room temperature for 15 min with gentle agitation. The reaction was stopped by removing the reaction mixture and adding to it a tube containing 3.9 ml of sodium buffer (i.e., a 40-fold dilution). The product was concentrated by a pre-wet Ultrafree centrifugal filter unit. The volume of concentrate was measured and 1/9 volume of 10 X salt solution (1 M NaCl, 0.2 M sodium citrate, 3 mM citric acid) was added. The protein concentration was determined, and the product recovery was then calculated. The products were analyzed by IEF gel followed by an *in vitro* test with TF-1 cells.

[00250]      2.      Another method for iodinating erythropoietin involves incubating one Iodo Bead (Pierce, Rockford, IL) in 100 ul PBS (20mM sodium phosphate, 0.15M NaCl, pH7.5)

containing 1 mCi free Na<sup>125</sup>I for 5 minutes. Erythropoietin (100 ug) in 100 ul PBS was then added to the mixture. After a ten minute incubation period at room temperature, the reaction was stopped by removing the 200 ul solution from the reaction vessel (leaving the iodo bead behind). The excess iodine was then removed by gel filtration on a Centricon 10 column. As shown in **Figure 9**, iodo-erythropoietin produced in this manner is efficacious in protecting P19 cells from serum withdrawal.

[00251] 3. Erythropoietin may also be iodinated using Chloramine T. Erythropoietin (100 ug) in 100 ul PBS was added to 500 uCi Na<sup>125</sup>I, and the mixture was then mixed together in an eppendorf tube. 25 ul chloramines T (2 mg/ml) were then added and the mixture was incubated for 1 minute at room temperature. 50 ul of Chloramine T stop buffer (2.4 mg/ml sodium metabisulfite, 10 mg/ml tyrosine, 10% glycerol, 0.1% xylene in PBS) was then added. The iodotyrosine and iodinated erythropoietin were then separated by gel filtration on a Centricon 10 column.

[00252] **G. Preparation of Tissue Protective Cytokine by Biotinylating Erythropoietin**

[00253] 1. In "Biotinylated recombinant human erythropoietins: Bioactivity and Utility as a receptor ligand" by Wojchowski et al. Blood, 1989, 74(3):952-8, the authors use three different methods of biotinylating erythropoietin. Biotin is added to (1) the sialic acid moieties (2) carboxylate groups and (3) amino groups. The authors use a mouse spleen cell proliferation assay to demonstrate that (1) the addition of biotin to the sialic acid moieties does not inactivate the biological activity of erythropoietin (2) the addition of biotin to carboxylate groups led to substantial biological inactivation of erythropoietin (3) the addition of biotin to amino groups resulted in complete biological inactivation of erythropoietin. These methods and

modifications are fully embraced herein. **Figure 10** shows the activity of biotinylated erythropoietin and asialoerythropoietin in the serum-starved P19 assay.

[00254] 2. Additionally, native erythropoietin may be used to prepare the respective biotinylated molecules, in accordance with the following procedure, as described in instruction provided by Pierce Chemical Company (Rockford, IL) for EZ-Link NHS-LC-Biotin (product # 21336).

[00255] Immediately before the reaction, EZ-Link NHS-LC-Biotin (pierce, 21336) in DMSO at 2 mg/ml was dissolved. The reaction was performed in a tube (17 x 100 mm) with total volume of 1 ml containing 50 mM sodium bicarbonate (pH 8.3). Erythropoietin and < 10% of EZ-Link NHS-LC-Biotin were added to generate a solution with a molar ratio of Biotin/protein at ~ 20. The solution was mixed well and incubated on ice for 2 h. The solution was desalted and concentrated using an Ultrafree centrifugal filter unit. The product was then collected into a fresh tube. The volume of the product was measured, and 1/9 volume of 10 X salt solution (1 M NaCl, 0.2 M sodium citrate, 3 mM citric acid) was added to the product. The protein content of the product was determined and the product recovery rate was calculated. The products were analyzed by IEF gel followed by an *in vitro* test with TF-1 cells.

[00256] 3. The free amino groups of erythropoietin can also be biotinylated using the following method. First, 0.2 mg D-biotinoyl-e-aminocaproic acid-N-hydroxysuccinimide ester (Boehringer Mannheim #1418165) was dissolved in 100 ul DMSO. This solution was then combined with 400 ul PBS containing approximately 0.2 mg erythropoietin in a foil covered tube. After incubating this solution for 4 hours at room temperature, the unreacted biotin was separated by gel filtration on a Centricon 10 column.

[00257] It is contemplated that several of these modifications may be performed on erythropoietin or an erythropoietin derivative in order to arrive at a tissue protective cytokine. For example erythropoietin can be desialylated in accordance with the procedure listed above at Example 2(A) and carbamylated in accordance with the procedure listed above at Example 2(B) to generate an asialo carbamoylerythropoietin.

[00258] **Example 3**

[00259] **PREPARATION OF TISSUE PROTECTIVE CYTOKINES BY OTHER METHODS**

[00260] 1. Trinitrophenylation: erythropoietin (100 ug) was modified with 2,4,6-trinitrobenzenesulfonate as described in Plapp et al ("Activity of bovine pancreatic deoxyribonuclease A with modified amino groups" 1971, J. Biol. Chem. 246, 939-845)

[00261] 2. Arginine modifications: erythropoietin was modified with 2,3-butanedione as described in Riordan ("Functional arginyl residues in carboxypeptidase A. Modification with butanedione" Riordan JF, Biochemistry 1973, 12(20): 3915-3923).

[00262] 3. Erythropoietin was modified with cyclohexanone as in Patthy et al ("Identification of functional arginine residues in ribonuclease A and lysozyme" Patthy, L, Smith EL, J. Biol. Chem 1975 250(2): 565-9).

[00263] 4. Erythropoietin was modified with phenylglyoxal as described in Werber et al. ("Proceedings: Carboxypeptidase B: modification of functional arginyl residues" Werber, MM, Sokolovsky M Isr J Med Sci 1975 11(11): 1169-70). The phenylglyoxal-modified erythropoietin was tested using the neural-like P19 cell assay described above. As **Figure 11**

illustrates, this chemically-modified erythropoietin fully retains its neuroprotective effects.

[00264] 5. Tyrosine modifications: erythropoietin (100 ug) was incubated with tetranitromethane as previously described in Nestler et al "Stimulation of rat ovarian cell steroidogenesis by high density lipoproteins modified with tetranitromethane" Nestler JE, Chacko GK, Strauss JF 3rd. J Biol Chem 1985 Jun 25;260(12):7316-21).

[00265] 6. Glutamic acid (and aspartic acid) modifications: In order to modify carboxyl groups, erythropoietin (100 ug) was incubated with 0.02 M EDC in 1M glycylamide at pH 4.5 at room temperature for 60 minutes as described in Carraway et al "Carboxyl group modification in chymotrypsin and chymotrypsinogen." Carraway KL, Spoerl P, Koshland DE Jr. J Mol Biol 1969 May 28;42(1):133-7.

[00266] 7. Tryptophan residue modifications: erythropoietin (100 ug) was incubated with 20 uM n-bromosuccinimide in 20 mM potassium phosphate buffer (pH 6.5) at room temperature as described in Ali et al., J Biol Chem. 1995 Mar 3;270(9):4570-4. The number of oxidized tryptophan residues was determined by the method described in Korotchkina (Korotchkina, LG et al Protein Expr Purif. 1995 Feb;6(1):79-90).

[00267] 8. Removal of amino groups: In order to remove amino groups of erythropoietin (100 ug) was incubated with in PBS (pH 7.4) containing 20mM ninhydrin (Pierce Chemical, Rockford, IL), at 37 C for two hours as in Kokkini et al (Kokkini, G., et al "Modification of hemoglobin by ninhydrin" Blood, Vol. 556, No 4 1980: 701-705). Reduction of the resulting aldehyde was accomplished by reacting the product with sodium borohydride or lithium aluminum hydride. Specifically, erythropoietin (100 ug) was incubated with 0.1M sodium borohydride in PBS for 30 minutes at room temperature. The reduction was terminated

by cooling the samples on ice for 10 minutes and dialyzing it against PBS, three times, overnight. (Kokkini, G., Blood, Vol. 556, No 4 1980: 701-705). Reduction using lithium aluminum hydride was accomplished by incubating erythropoietin (100 ug) with 0.1M lithium aluminum hydride in PBS for 30 minutes at room temperature. The reduction was terminated by cooling the samples on ice for 10 minutes and dialyzing the samples against PBS, three times, overnight.

[00268] 9. Disulfide reduction and stabilization: erythropoietin (100 ug) was incubated with 500 mM DTT for 15 minutes at 60 C. 20 mM iodoacetamide in water was then added to the mixture and incubated for 25 minutes, at room temperature in the dark.

[00269] 10. Limited proteolysis: Erythropoietin can be subjected to a limited chemical proteolysis that targets specific residues. Erythropoietin was reacted with 2-(2-nitrophenylsulfenyl)-3-methyl-3'-bromoindolenine which cleaves specifically after tryptophan residues in a 50 times excess in 50% acetic acid for 48 hours in the dark at room temperature in tubes capped under nitrogen pressure. The reaction was terminated by quenching with tryptophan and desalting.

[00270] As noted above, erythropoietin or asialoerythropoietin may be modified, yet multiple modifications as well as additional modifications of the erythropoietin molecule may also be performed without deviating from the spirit of the present invention. Any of the foregoing examples may be carried out with partially desialylated erythropoietin, which may be prepared as described below. For example, any of the aforementioned modified erythropoietins may be modified at one or more arginine residues by using, for example, phenylglyoxal according to the protocol of Takahashi (1977, *J. Biochem.* 81:395-402), which may be carried out for variable lengths of time ranging from 0.5 to 3 hrs at room temperature. The reaction was

terminated by dialyzing the reaction mixture against water. Use of such modified forms of erythropoietin is fully embraced herein.

#### Example 4

#### [00271] TISSUE PROTECTIVE CYTOKINES HAVE NEURO PROTECTIVE EFFECT

[00272] The neuroprotective affects of the tissue protective cytokines of the present invention was evaluated using a water intoxication assay in accordance with Manley et al., 2000, Aquaporin-4 deletion in mice reduces brain edema after acute water intoxication and ischemic stroke, Nat Med 2000 Feb;6(2):159-63. Female C3H/HEN mice were used. The mice were given 20% of their body weight as water IP with 400 ng/kg bw DDAVP (desmopressin). The mice were administered erythropoietin (A) or a tissue protective cytokine: asialoerythropoietin (B), carbamylated asialoerythropoietin (C), succinylated asialoerythropoietin (D), acetylated asialoerythropoietin (E), iodinated asialoerythropoietin (F), carboxymethylated asialoerythropoietin (G), carbamylated erythropoietin (H), acetylated erythropoietin (I), iodinated erythropoietin (J), or N<sup>ε</sup>-carboxy methyl erythropoietin (K). The mice were given a 100 microgram/kg dose of erythropoietin or tissue protective cytokine intraperitoneally 24 hrs before administration of the water and again at the time of the water administration. A modified scale from Manley et al. was used to evaluate the mice. The modified scale is as listed below:

##### 1. Explores cage/table

|     |   |
|-----|---|
| Yes | 0 |
|-----|---|

|    |   |
|----|---|
| No | 1 |
|----|---|

##### 2. Visually tracks objects

|     |   |
|-----|---|
| Yes | 0 |
|-----|---|

|                                |   |
|--------------------------------|---|
| No                             | 1 |
| 3. Whisker movement            |   |
| Present                        | 0 |
| Absent                         | 1 |
| 4. Leg-tail movements          |   |
| Normal                         | 0 |
| Stiff                          | 1 |
| Paralyzed                      | 2 |
| 5. Pain withdrawal (toe pinch) |   |
| Yes                            | 0 |
| No                             | 1 |
| 6. Coordination of movement    |   |
| Normal                         | 0 |
| Abnormal                       | 1 |
| 7. Stops at edge of table      |   |
| Yes                            | 0 |
| No                             | 1 |

Total score possible : 8

[00273] The mice were scored at the following time points: 15, 30, 45, 60, 75, 90, 120, 150, 180 minutes. Score as plotted is the area under the entire time curve, as percent of animals that had received saline only. **Figure 12** shows the scores of the mice who received erythropoietin or one of the tissue protective cytokines of the present invention as a percentage of the score obtained by the control mice. The mice who received the tissue protective cytokines

of the present invention exhibited less neurological damage and therefore scored better on the modified scale. **Figure 12** shows that the tissue protective cytokines of the present invention protect neuronal tissue.

[00274] **Example 5**

[00275] **ERYTHROPOIETIN CROSSES THE BLOOD-CEREBROSPINAL FLUID  
TIGHT BARRIER**

[00276] Adult male Sprague-Dawley rats were anesthetized and administered recombinant human erythropoietin intraperitoneally. Cerebrospinal fluid was sampled from the cisterna magna at 30 minute intervals up to 4 hrs and the erythropoietin concentration determined using a sensitive and specific enzyme-linked immunoassay. As illustrated in **Figure 13**, the baseline erythropoietin concentration in CSF is 8 mU/ml. After a delay of several hours, the levels of erythropoietin measured in the CSF begin to rise and by 2.5 hours and later are significantly different from the baseline concentration at the  $p < 0.01$  level. The peak level of about 100 mU/ml is within the range known to exert protective effects *in vitro* (0.1 to 100 mU/ml). The time to peak occurs at about 3.5 hrs, which is delayed significantly from the peak serum levels (less than 1 hr). The results of this experiment illustrate that significant levels of erythropoietin can be accomplished across a tight cellular junction by bolus parenteral administration of erythropoietin at appropriate concentrations. One of ordinary skill in the art would recognize that similar results would be expected from the tissue protective cytokines of the present invention.

[00277] **Example 6**

[00278] **MAINTENANCE OF FUNCTION IN HEART PREPARED FOR  
TRANSPLANTATION**

[00279] Wistar male rats weighing 300 to 330g are given erythropoietin (5000 U/kg body weight) or vehicle 24h prior to removal of the heart for *ex vivo* studies, done in accordance with the protocol of Delcayre et al., 1992, *Amer. J. Physiol.* 263:H1537-45. Animals are sacrificed with pentobarbital (0.3mL), and intravenously heparinized (0.2mL). The hearts are initially allowed to equilibrate for 15 min. The left ventricular balloon is then inflated to a volume that gives an end-diastolic pressure of 8 mm Hg. A left ventricular pressure-volume curve is constructed by incremental inflation of the balloon volume by 0.02 ml aliquots. Zero volume is defined as the point at which the left ventricular end-diastolic pressure is zero. On completion of the pressure-volume curve, the left ventricular balloon is deflated to set end-diastolic pressure back to 8mmHg and the control period is pursued for 15 min., after check of coronary flow. Then the heart is arrested with 50 mL Celsior + molecule to rest at 4°C under a pressure of 60cm H<sub>2</sub>O. The heart is then removed and stored 5 hours at 4°C in plastic container filled with the same solution and surrounded with crushed ice.

[00280] On completion of storage, the heart is transferred to a Langendorff apparatus. The balloon catheter is re-inserted into the left ventricle and re-inflated to the same volume as during preischemic period. The heart is re-perfused for at least 2 hours at 37°C. The re-perfusion pressure is set at 50cm H<sub>2</sub>O for 15min of re-flow and then back to 100cm H<sub>2</sub>O for the 2 next hours. Pacing (320 beats per minute) is re-instituted. Isovolumetric measurements of contractile indexes and diastolic pressure are taken in triplicate at 25, 45, 60, 120 min of reperfusion. At this time point pressure volume curves are performed and coronary effluent during the 45min reperfusion collected to measure creatine kinase leakage. The two treatment groups are compared using an unpaired t-test, and a linear regression using the end-diastolic pressure data is used to design compliance curves. As shown in **Figure 14**, significant improvement of left ventricular pressure developed occurs after treatment with erythropoietin, as well as improved

volume-pressure curve, decrease of left diastolic ventricular pressure and decrease of creatine kinase leakage. Similar results would be expected from treatment with the tissue protective cytokines of the present invention.

[00281] **Example 7**

[00282] **ERYTHROPOIETIN PROTECTS MYOCARDIUM FROM ISCHEMIC INJURY**

[00283] Adult male rats given recombinant human erythropoietin (5000 U (40 µg)/kg body weight) 24 hrs previously are anesthetized and prepared for coronary artery occlusion. An additional dose of erythropoietin is given at the start of the procedure and the left main coronary artery occluded for 30 minutes and then released. The same dose of erythropoietin is given daily for one week after treatment. The animals are then studied for cardiac function. As **Figure 15** illustrates, animals receiving a sham injection (saline) demonstrated a large increase in the left end diastolic pressure, indicative of a dilated, stiff heart secondary to myocardial infarction. In contradistinction, animals receiving erythropoietin suffered no decrement in cardiac function, compared to sham operated controls (difference significant at the  $p < 0.01$  level). Similar results would be expected from treatment with the tissue protective cytokines of the present invention.

[00284] **Example 8**

[00285] **PROTECTION OF RETINAL ISCHEMIA BY PERIPHERALLY-ADMINISTERED ERYTHROPOIETIN.**

[00286] Retinal cells are very sensitive to ischemia such that many will die after 30

minutes of ischemic stress. Further, subacute or chronic ischemia underlies the deterioration of vision which accompanies a number of common human diseases, such as diabetes mellitus, glaucoma, and macular degeneration. At the present time there are no effective therapies to protect cells from ischemia. A tight endothelial barrier exists between the blood and the retina that excludes most large molecules. To test whether peripherally-administered erythropoietin will protect cells sensitive to ischemia, an acute, reversible glaucoma rat model was utilized as described by Rosenbaum et al. (1997; *Vis. Res.* 37:3443-51). Specifically, saline was injected into the anterior chamber of the eye of adult male rats to a pressure above systemic arterial pressure and maintained for 60 minutes. Animals were administered saline or 5000 U (40  $\mu$ g) erythropoietin/kg body weight intraperitoneally 24 hours before the induction of ischemia, and continued as a daily dose for 3 additional days. Electroretinography was performed on dark-adapted rats 1 week after treatment. **Figure 16-17** illustrate that the administration of erythropoietin is associated with good preservation of the electroretinogram (ERG) (Panel D), in contrast to animals treated with saline alone (Panel C), for which very little function remained. **Figure 16** compares the electroretinogram a- and b-wave amplitudes for the erythropoietin-treated and saline-treated groups, and shows significant protection afforded by erythropoietin. Similar results are obtainable from treatment with the tissue protective cytokines of the present invention.

[00287]      **Example 9**

[00288]      **RESTORATIVE EFFECTS OF ERYTHROPOIETIN ON DIMINISHED  
COGNITIVE FUNCTION ARISING FROM BRAIN INJURY**

[00289]      In a study to demonstrate the ability of erythropoietin to restore diminished cognitive function in mice after receiving brain trauma, female Balb/c mice were subject to blunt brain trauma as described in Brines et al. PNAS 2000, 97; 10295-10672 and five days later, daily erythropoietin administration of 5000 U (40  $\mu$ g)/kg-bw intraperitoneally was begun. Twelve days after injury, animals were tested for cognitive function in the Morris water maze, with four trials per day. While both treated and untreated animals performed poorly in the test (with swim times of about 80 seconds out of a possible 90 seconds), **Figure 18** shows that the erythropoietin-treated animals performed better (in this presentation, a negative value is better). Even if the initiation of erythropoietin treatment is delayed until 30 days after trauma (**Figure 19**), restoration of cognitive function is also seen. Similar results would be expected from treatment with the tissue protective cytokines of the present invention.

[00290]      **Example 10**

[00291]      **KAINATE MODEL**

[00292]      In the kainate neurotoxicity model, asialoerythropoietin was administered according to the protocol of Brines et al. Proc. Nat. Acad. Sci. U.S.A. 2000, 97; 10295-10672 at a dose of 5000U (40  $\mu$ g)/kg-bw given intraperitoneally 24 hours before the administration of 25 mg/kg kainate is shown to be as effective as erythropoietin, as shown by time to death (**Figure 20**). Similar results are obtainable from treatment with the tissue protective cytokines of the present invention.

[00293]      **Example 11**[00294]      **SPINAL CORD INJURY MODELS**

[00295]

[00296]      1.      Rat Spinal Cord Compression Testing Erythropoietin and Tissue Protective Cytokines

[00297]      Wistar rats (female) weighing 180–300g were used in this study. The animals were fasted for 12 h before surgery, and were humanely restrained and anesthetized with an intraperitoneal injection of thiopental sodium (40 mg/kg-bw). After infiltration of the skin (bupivacaine 0.25%), a complete single level (T-3) laminectomy was performed through a 2 cm incision with the aid of a dissecting microscope. Traumatic spinal cord injury was induced by the extradural application of a temporary aneurysm clip exerting a 0.6 newton (65 grams) closing force on the spinal cord for 1 minute. After removal of the clip, the skin incision was closed and the animals allowed to recover fully from anesthesia and returned to their cages. The rats were monitored continuously with bladder palpation at least twice daily until spontaneous voiding resumed.

[00298]      40 animals were randomly divided into five groups. Animals in the control group (I) ( $n = 8$ ) received normal saline (via intravenous injection) immediately after the incision is closed. Group (II;  $n = 8$ ) received rhEPO @ 16 micrograms/kg-bw iv; group (III) received an asialo tissue protective cytokine of the present invention (asialoerythropoietin) @ 16 micrograms/kg-bw iv, group (IV) received an asialo tissue protective cytokine @ 30 micrograms/kg-bw iv, and group (V) received an asialo tissue protective cytokine of the present invention (asialoerythropoietin) @ 30 micrograms/kg-bw; all as a single bolus intravenous injection immediately after removal of the aneurysm clip.

[00299]      Motor neurological function of the rats will be evaluated by use of the locomotor rating scale of Basso et al. In this scale, animals are assigned a score ranging from 0 (no

observable hindlimb movements) to 21 (normal gait). The rats will be tested for functional deficits at 1, 12, 24, 48, 72 hours and then at 1 week after injury by the same examiner who is blind to the treatment each animal receives.

[00300] **Figure 21** is a graph demonstrating the locomotor ratings of the rats recovering from the spinal cord trauma over a period of thirty days. As can be seen from the graph, the rats that were given erythropoietin (group II) or tissue protective cytokines (groups III-V) recovered from the injury more readily and demonstrated better overall recovery from the injury than the control rats. Similar results would be expected from the therapeutic treatment with the tissue protective cytokines of the present invention.

[00301] 2. Rabbit Spinal Cord Ischemia Testing Erythropoietin and a Tissue Protective Cytokine.

[00302] 36 New Zealand White rabbits (8-12 months old, male) weighing 1.5-2.5 kg were used in this study. The animals were fasted for 12 hours and humanely restrained. Anesthesia induction was via 3 % halothane in 100 % oxygen and maintained with 0.5 – 1.5 % halothane in a mixture of 50 % oxygen and 50 % air. An intravenous catheter (22 gauge) was placed in the left ear vein. Ringers lactate was infused at a rate of 4 ml/kg body weight (bw) per hour during the surgical procedure. Preoperatively, cefazoline 10 mg/kg-bw was administered intravenously for prophylaxis of infection. The animals were placed in the right lateral decubitus position, the skin prepared with povidone iodine, infiltrated with bupivacaine (0.25 %) and a flank skin incision was made parallel to the spine at the 12th costal level. After incision of the skin and subcutaneous thoracolumbar fascia, the longissimus lumborum and iliocostalis lumborum muscles were retracted. The abdominal aorta was exposed via a left retroperitoneal approach and mobilized just inferior to the left renal artery. A piece of PE-60 tubing was looped around the

aorta immediately distal to the left renal artery and both ends passed through a larger rubber tube. By pulling on the PE tubing, the aorta was non-traumatically occluded for 20 minutes. Heparin (400 IU) was administered as an intravenous bolus before aortic occlusion. After 20 minutes of occlusion, the tube and catheter were removed, the incision was closed and the animals were monitored until full recovery and then were serially assessed for neurological function.

[00303] 36 animals were randomly divided into six groups. In a control group (I), animals (n = 6) received normal saline intravenously immediately after release of aortic occlusion. Group (II) received rhEPO @ 6.5 microgram/kg-bw; group (III) received a tissue protective cytokine (carbamylated erythropoietin) @ 6.5 microgram/kg-bw; group (IV) received another tissue protective cytokine (asialoerythropoietin) @ 6.5 microgram/kg-bw; group (V) received the same tissue protective cytokine as group (IV) but @ 20 microgram/kg-bw; and group (VI) received yet another tissue protective cytokine (asialocarbamylatederythropoietin) @ 20 microgram/kg-bw all intravenously immediately after reperfusion (n = 6 for each group).

[00304] Motor function was assessed according to the criteria of Drummond and Moore by an investigator blind to the treatment at 1, 24 and 48 h after reperfusion. A score of 0 to 4 was assigned to each animal as follows: 0 = paraplegic with no evident lower extremity motor function; 1 = poor lower extremity motor function, weak antigravity movement only; 2 = moderate lower extremity function with good antigravity strength but inability to draw legs under body; 3 = excellent motor function with the ability to draw legs under body and hop, but not normally; 4 = normal motor function. The urinary bladder was evacuated manually in paraplegic animals twice a day.

[00305] **Figure 22** is a graph plotting motor function of the recovering rabbits. The graph demonstrates that even over a period of only two days erythropoietin and the tissue protective cytokines of the present invention permit the rabbits to recover more fully from the spinal cord injury. Similar results would be expected from the therapeutic treatment with the tissue protective cytokines of the present invention.

[00306] **Example 12**

[00307] **ANTI-INFLAMMATORY AFFECTS OF ERYTHROPOIETIN**

[00308] **In-Vivo Studies:**

[00309] 1. **Middle Cerebral Artery Occlusion (MCAO) Studies on Rats.**

[00310] Male Crl:CD(SD)BR rats weighing 250-280 g were obtained from Charles River, Calco, Italy. Surgery was performed on these rats in accordance with the teachings of Brines, M.L., Ghezzi, P., Keenan, S., Agnello, D., de Lanerolle, N.C., Cerami, C., Itri, L.M., and Cerami, A. 2000 Erythropoietin crosses the blood-brain barrier to protect against experimental brain injury [In Process Citation] Proc Natl Acad Sci USA 97:10526-10531. Briefly, the rats were anesthetized with chloral hydrate (400 mg/kg-bw, i.p.), the carotid arteries were visualized, and the right carotid was occluded by two sutures and cut. A burr hole adjacent and rostral to the right orbit allowed visualization of the MCA, which was cauterized distal to the rhinal artery. To produce a penumbra (borderzone) surrounding this fixed MCA lesion, the contralateral carotid artery was occluded for 1 hour by using traction provided by a fine forceps and then re-opened. PBS or rhEPO (5,000 U/kg-bw, i.p.; previously shown to be protective in this model (1)) were administered immediately after the MCAO. When indicated, TNF and IL-6 were

quantified in brain cortex homogenates as previously described (8). MCP-1 was measured in the homogenates using a commercially available ELISA kit (biosource, Camarillo, CA).

[00311] Twenty-four hours after MCAO, the rats were anesthetized as described above and transcardially perfused with 100ml saline followed by 250 ml of sodium phosphate buffered 4% paraformaldehyde solution. Brains were rapidly removed, fixed in sodium phosphate buffered 4 % paraformaldehyde solution for two hours, transferred to 20% sucrose solution in PBS overnight, then in 30% sucrose solution until they sank and were then frozen in 2-methylbutane at -45° C. Sections (30  $\mu$ m) were cut on a cryostat (HM 500 OM, Microm) at -20° C in the transverse plane through the brain and selected every fifth section for histochemistry against the different antigens, or hematoxylin-eosin staining. Free floating sections were processed for immunoreactivity both with anti-glial fibrillary acid protein (GFAP) mouse monoclonal antibody (1:250, Boehringer Mannheim, Monza, Italy) and with anti-cd11b (MRC OX-42) mouse monoclonal antibody (1:50, Serotec, UK), according to the protocols described by Houser et al. and the manufacturer's protocol respectively. All sections were mounted for light microscopy in saline on coated slides, dehydrated through graded alcohols, fixed in xylene and coverslipped using DPX mountant (BDH, Poole, UK). Adjacent sections were stained with hematoxylin-eosin as described (10).

[00312] **Figure 23** shows a coronal section of the brain cortical layer stained by hematoxilyn and eosin. Control rat (A), ischemic rat treated with PBS (B), ischemic rat treated with rhEPO (5,000 U/kg-bw, i.p., immediately following MCAO) (C). The section B shows a marked decrease in tissue staining consistent with inflammation, accompanied by a loss of neuronal component compared to the control (A). Systemic rhEPO administration largely reduces the ischemic damage localizing the cell death or injury in a restricted area (C). (Magnification 2.5x. Size bar = 800  $\mu$ m.)

[00313] **Figure 24** shows coronal sections of frontal cortex adjacent to the region of infarction stained by GFAP antibody. Control rat (A), ischemic rat treated with PBS (B), ischemic rat treated with rhEPO (C). Activated astrocytes are visualized by their GFAP-positive processes (Panel B). There was a marked reduction in number as well as staining intensity of activated astrocytes in a representative rhEPO-treated animal (Panel C). (Magnification 10x. Size bar = 200  $\mu\text{m}$ .)

[00314] **Figure 25** shows coronal sections of brain cortical layer stained by OX-42 antibody. Ischemic rat treated with PBS (A), ischemic rat treated with rhEPO (B). In the ischemic cerebral hemisphere the cellular staining is especially prominent around the infarcted tissue in both treatment groups, but is much denser and extends further in the saline treated group. (Magnification 20x; Size bar = 100  $\mu\text{m}$ ).

[00315] **Figure 26** shows coronal sections of brain cortical layer adjacent to the region of infarction stained by OX-42 antibody. A much higher density of mononuclear inflammatory cells are observed in the tissue from an ischemic rat treated with PBS (A) compared to an ischemic rat treated with rhEPO (B). The infiltrating leukocytes, with typical round shape, potentially will extend the volume of infarction. (Magnification 10x; Size bar = 200  $\mu\text{m}$ )

[00316] Similar results would be expected from the therapeutic treatment with the tissue protective cytokines of the present invention.

[00317] 2. Acute Experimental Allergic Encephalomyelitis (EAE) in Lewis rats

[00318] Female Lewis rats, 6-8 weeks of age, were purchased from Charles River (Calco, Italy). EAE was induced in rats by injecting 50  $\mu\text{g}$  of guinea pig MBP (Sigma, St. Louis, MO)

in water emulsified in equal volumes of complete Freund's adjuvant (CFA, Sigma) added with 7 mg/ml of heat-killed *M. tuberculosis* H37Ra (Difco, Detroit, MI) in a final volume of 100  $\mu$  under light ether anesthesia into both hind footpads. Rats were examined in a blinded fashion for signs of EAE and scored as follows: 0, no disease; 1, flaccid tail; 2, ataxia; 3, complete hind limb paralysis with urinary incontinence. Starting from day 3 after immunization, rats were given r-Hu-EPO (EPOetin alfa, Procrit, Ortho Biotech, Raritan, NJ) intraperitoneally (i.p.) once a day at the indicated doses, in PBS. Since the clinical-grade EPO contained human serum albumin, control animals were always given PBS containing an identical amount of human serum albumin. Daily administration of 5,000 U/kg-bw of EPO increased the hematocrit by 30% (data not shown). When indicated, rats were injected s.c. once a day from day 3 until day 18 with 1.3 mg/kg-bw dexamethasone (DEX) phosphate disodium salt (Sigma) corresponding to 1 mg/kg-bw of DEX, dissolved in PBS. When indicated, TNF and IL-6 were quantified in brain and spinal cord homogenates as previously described [Agnello, 2000 #10].

[00319] **Figure 27** shows the protective effect on the clinical signs of EAE of different doses of EPO, given from day 3 after immunization with MBP until day 18. EPO, in a dose-dependent fashion, delayed the onset of disease and decreased disease severity. But, EPO did not delay the time to greatest severity.

[00320] In experiments where treatment of EPO was discontinued after the disease regressed and the rats were monitored up to two months, no relapse was observed, in contrast with DEX which induces an exacerbation of disease after suspending its administration (**Figure 28**). Similar results would be expected from the therapeutic treatment with the tissue protective cytokines of the present invention.

[00321]      In Vitro Studies:

[00322]      Primary cultures of glial cells were prepared from new born Sprague-Dawley rats 1-2 days old. Cerebral hemispheres were freed from the meninges and mechanically disrupted. Cells were dispersed in a solution of trypsin 2.5% and DNAase 1%, filtered through a 100  $\mu$ m nylon mesh and plated (140,000 cells per 35 mm dish) in Eagle's minimum essential medium supplemented with 10% fetal calf serum, 0.6% glucose, streptomycin (0.1 mg/ml) and penicillin (100 U/ml). Glial cultures were fed twice a week and grown at 37° C in a humidified incubator with 5% CO<sub>2</sub>. All experiments were performed on 2-3 week-old glial cell cultures with 97% astrocytes and 3% microglia, as assessed by immunocytochemistry oGFAP and *Griffonia simplicifolia* isolectin B<sub>4</sub>. Neuronal cultures were established from the hippocampus of 18-day rat fetuses. Brains were removed and freed from meninges and the hippocampus was isolated. Cells were dispersed by incubation for 15-20 min at 37° C in a 2.5% trypsin solution followed by titration. The cell suspension was diluted in the medium used for glial cells and plated onto polyornithine-coated coverslips at a density of 160,000 cells per coverslip. The day after plating, coverslips were transferred to dishes containing a glial monolayer in neuron maintenance medium (Dulbecco's modified Eagle's medium and Ham's nutrient mix F12 supplemented with 5  $\mu$ g/ml insulin, 100  $\mu$ g/ml transferrin, 100  $\mu$ g/ml putrescin, 30 nM Na selenite, 20 nM progesterone and penicillin 100 U/ml) supplemented with cytosine arabinoside 5  $\mu$ M. Coverslips were inverted so that the hippocampal neurons faced the glia monolayer. Paraffin dots adhering to the coverslips supported them above the glia, creating a narrow gap that prevented the two cell types from contacting each other but allowed the diffusion of soluble substances. These culture conditions allowed the growth of differentiated neuronal cultures with >98% homogeneity, as assessed by immunocytochemistry of microtubule-associated protein 2 and GFAP. Cells were then treated for 24 hours with 1

$\mu$ M Trimethyl tin (TMT), in the presence or absence of rhEPO (10 U (80 ng)/ml), the supernatants used for TNF assay and cellular viability evaluated as described below. When indicated, glial cells were cultured in the presence of LPS for 24 hours, with or without rhEPO, and TNF measured in the cultured supernatants. Cell viability was measured by the 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Denizot, F., and Lang, R. 1986. Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J Immunol Methods* 89:271-277. Briefly, MTT tetrazolium salt was dissolved in serum-free medium to a final concentration of 0.75 mg/ml and added to the cells at the end of the treatment for 3 h at 37° C. The medium was then removed and the formazan was extracted with IN HCl:isopropanol (1:24). Absorbance at 560 nm was read on a microplate reader.

[00323] **Figure 29** shows that rhEPO prevents neuronal death-induced TNF production in mixed neuron-glia cultures. Panel A: Percentage of neural cell death induced by TMT 1  $\mu$ M without or with treatment with rhEPO (10 U/ml). Panel B: Release of TNF- $\alpha$  from glial cells exposed to TMT 1  $\mu$ M in the presence (hatched bars) or absence (filled bars) of neurons, with or without rhEPO (10 U/ml). Similar results would be expected from the therapeutic treatment with the tissue protective cytokines of the present invention.

[00324] The invention is not to be limited in scope by the specific embodiments described which are intended as single illustrations of individual aspects of the invention, and functionally equivalent methods and components are within the scope of the invention. Indeed various modifications of the invention, in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

[00325] All references cited herein are incorporated by reference herein in their entireties for all purposes.

**WHAT IS CLAIMED IS:**

1. A pharmaceutical composition comprising a therapeutically effective amount of a tissue protective cytokine; at least one anti-inflammatory agent; and a pharmaceutically acceptable carrier.
2. The pharmaceutical composition of claim 1, wherein the anti-inflammatory agent is selected from the group consisting of corticosteroids, glucocorticoids, steroids, non-steroidal anti-inflammatory drugs, beta-agonists, anticholinergic agents, methyl xanthines, gold injections, sulphasalazine, penicillamine, anti-angiogenic agents, dapsone, psoralens, anti-malarial agents, anti-viral agents, and antibiotics.
3. A pharmaceutical composition comprising a therapeutically effective amount of a tissue protective cytokine; at least one immunomodulatory agent; and a pharmaceutically acceptable carrier.
4. The pharmaceutical composition of claim 3, wherein the anti-inflammatory agent is selected from the group consisting of methothrexate, leflunomide, cyclophosphamide, cytoxan, Immuran, cyclosporine A, minocycline, azathioprine, antibiotics, methylprednisolone, corticosteroids, steroids, mycophenolate mofetil, rapamycin, mizoribine, deoxyspergualin, brequinar, malononitriloamides, T cell receptor modulators, and cytokine receptor modulators.
5. A pharmaceutical composition of claim 1 or 3, wherein said tissue protective cytokine is selected from the group consisting of i) an erythropoietin that lacks sialic acid moieties; ii) an erythropoietin that lacks N-linked or lacks O-linked carbohydrates; iii) an erythropoietin having a reduced carbohydrate content by treatment of native erythropoietin with at least one glycosidase; iv) an erythropoietin having at least one or more oxidized carbohydrates; v) an erythropoietin comprising at least one or more oxidized carbohydrates which is chemically reduced; vi) an erythropoietin comprising at least one or more modified arginine residues; vii) an erythropoietin comprising at least one or more modified lysine residues or a modification of

the N-terminal amino group of the erythropoietin molecule; viii) an erythropoietin comprising at least a modified tyrosine residue; ix) an erythropoietin comprising at least a modified aspartic acid or a glutamic acid residue; x) an erythropoietin comprising at least a modified tryptophan residue; xi) an erythropoietin having at least one amino group removed; xii) an erythropoietin comprising at least an opening of at least one of the cystine linkages in the erythropoietin molecule; and xiii) a truncated erythropoietin.

6. A method for treating inflammation in a mammal comprising responsive cells, tissues, and/or organs, said method comprising administering to a mammal a pharmaceutical composition comprising a therapeutically effective amount of a tissue protective cytokine and a pharmaceutically acceptable carrier.

7. The method of Claim 6, wherein the tissue protective cytokine lacks at least one activity selected from the group consisting of increasing hematocrit, vasoconstriction, hyperactivating platelets, pro-coagulant activity and increasing production of thrombocytes.

8. A method treating inflammation in a mammal comprising responsive cells, tissues, and/or organs, said method comprising administering to a mammal in need thereof a pharmaceutical composition comprising a prophylactically or therapeutically effective amount of a tissue protective cytokine and a pharmaceutically acceptable carrier, and administering to the mammal a prophylactically or therapeutically effective amount of one or more anti-inflammatory agents or immunomodulatory agents.

9. The method of claim 8, wherein the anti-inflammatory agent is selected from the group consisting of a corticosteroid, a glucocorticoid, a steroid, a non-steroidal anti-inflammatory drug, a beta-agonist, a anticholinergic agent, a methyl xanthine, gold injection, a sulphasalazine, penicillamine, a anti-angiogenic agent, dapsone, psoralen, a anti-malarial agent, a anti-viral agent, and an antibiotic.

10. The method of claim 8, wherein the immunomodulatory agent is selected from the group consisting of a proteinaceous agent, a peptide mimetic, an antibody, a nucleic acid molecule, a small molecule, an organic compound, an inorganic compound, methothrexate, leflunomide, cyclophosphamide, cytoxan, Immuran, cyclosporine A, minocycline, azathioprine, an antibiotic, methylprednisolone (MP), a corticosteroid, a steroid, mycophenolate mofetil, rapamycin, mizoribine, deoxyspergualin, brequinar, a malononitriloaminde, a T cell receptor modulator, and a cytokine receptor modulator.

11. The method of claim 6 or 8, wherein said tissue protective cytokine is i) an erythropoietin that lacks sialic acid moieties; ii) an erythropoietin that lacks N-linked or lacks O-linked carbohydrates; iii) an erythropoietin having a reduced carbohydrate content by treatment of native erythropoietin with at least one glycosidase; iv) an erythropoietin having at least one or more oxidized carbohydrates; v) an erythropoietin comprising at least one or more oxidized carbohydrates which is chemically reduced; vi) an erythropoietin comprising at least one or more modified arginine residues; vii) an erythropoietin comprising at least one or more modified lysine residues or a modification of the N-terminal amino group of the erythropoietin molecule; viii) an erythropoietin comprising at least a modified tyrosine residue; ix) an erythropoietin comprising at least a modified aspartic acid or a glutamic acid residue; x) an erythropoietin comprising at least a modified tryptophan residue; xi) an erythropoietin having at least one amino group removed; xii) an erythropoietin comprising at least an opening of at least one of the cystine linkages in the erythropoietin molecule; and xiii) a truncated erythropoietin.

12. The method of claim 6 or 8, or wherein said tissue protective cytokine is asialoerythropoietin or phenylglyoxal-erythropoietin.

13. The method of claim 6 or 8, wherein the tissue protective cytokine is capable of traversing an endothelial cell barrier.

14. The method of claim 13, wherein the endothelial cell barrier is selected from the group consisting of blood-brain barrier, blood-eye barrier, blood-testis barrier, blood-ovary barrier, and blood-uterus barrier.
15. The method of claim 6 or 8, wherein responsive cells, tissues, and/or organs in the mammal, are selected from the group consisting of neuronal cells, muscle cells, heart, lung, liver, kidney, small intestine, adrenal cortex, adrenal medulla, capillary cells, endothelial cells, testes, ovary, endometrial cells, and stem cells.
16. The method of claim 6 or 8, wherein the responsive mammalian cells further comprise cells selected from the group consisting of photoreceptor cells, ganglion cells, bipolar cells, horizontal cells, amacrine cells, Müller cells, myocardium cells, pace maker cells, sinoatrial node cells, sinoatrial node cells, sinus node cells, atrioventricular node cells, bundle of His cells, hepatocyte cells, stellate cells, Kupffer cells, mesangial cells, goblet cells, intestinal gland cells, enteral endocrine cells, glomerulosa cells, fasciculate cells reticularis cells, chromaffin cells, pericyte cells, Leydig cells, Sertoli cells, sperm cells, Graffian follicle cells, primordial follicle cells, endometrial stroma cells, and endometrial cells.
17. The method of claim 6 or 8, wherein said tissue protective cytokine is asialoerythropoietin.
18. The method of claim 17, wherein said asialoerythropoietin is human asialoerythropoietin.
19. The method of claim 6 or 8, wherein said tissue protective cytokine is an erythropoietin with no N-linked carbohydrates.
20. The method of claim 6 or 8, wherein said tissue protective cytokine is an erythropoietin with no O-linked carbohydrates.

21. The method of claim 6 or 8, wherein said tissue protective cytokine is an erythropoietin treated with at least one glycosidase.
22. The method of claim 6 or 8, wherein said tissue protective cytokine is periodate-oxidized erythropoietin.
23. The method of claim 22, wherein said periodate-oxidized erythropoietin is chemically reduced with sodium cyanoborohydride.
24. The method of claim 6 or 8, wherein said tissue protective cytokine is an erythropoietin comprising a R-glyoxal moiety on the one or more arginine residues, wherein R is aryl or alkyl moiety.
25. The method of claim 24, wherein said erythropoietin is phenylglyoxal-erythropoietin.
26. The method of claim 6 or 8, wherein said tissue protective cytokine is an erythropoietin in which at least one arginine residue is modified by reaction with a vicinal diketone selected from the group consisting of 2,3-butanedione and cyclohexanedione.
27. The method of claim 6 or 8, wherein said tissue protective cytokine is an erythropoietin in which at least one arginine residue is reacted with 3-deoxyglucosone.
28. The method of claim 6 or 8, wherein said tissue protective cytokine is an erythropoietin molecule comprising at least one biotinylated lysine or N-terminal amino group.
29. The method of claim 28, wherein said erythropoietin molecule is biotinylated erythropoietin.
30. The method of claim 6 or 8, wherein said tissue protective cytokine is a glucitolyl lysine erythropoietin or a fructosyl lysine erythropoietin.

31. The method of claim 6 or 8, wherein said tissue protective cytokine is an erythropoietin having at least one carbamylated lysine residue.
32. The method of claim 31, wherein said carbamylated erythropoietin is selected from the group consisting of alpha-N-carbamoylerythropoietin; N-epsilon-carbamoylerythropoietin; alpha-N-carbamoyl, N-epsilon-carbamoylerythropoietin; alpha-N-carbamoylasialoerythropoietin; N-epsilon-carbamoylasialoerythropoietin; alpha-N-carbamoyl, N-epsilon-carbamoylasialoerythropoietin; alpha-N-carbamoylhyposialoerythropoietin; N-epsilon-carbamoylhyposialoerythropoietin; and alpha-N-carbamoyl, N-epsilon-carbamoylhyposialoerythropoietin.
33. The method of claim 6 or 8, wherein said tissue protective cytokine is an erythropoietin in which at least one lysine residue is acylated.
34. The method of claim 33, wherein a lysine residue of said erythropoietin is acetylated.
35. The method of claim 34, wherein said acetylated erythropoietin is selected from the group consisting of alpha-N-acetylerythropoietin; N-epsilon-acetylerythropoietin; alpha-N-acetyl, N-epsilon-acetylerythropoietin; alpha-N-acetylalioerythropoietin; N-epsilon-acetylalioerythropoietin; alpha-N-acetyl, N-epsilon-acetylalioerythropoietin; alpha-N-acetylhyposialoerythropoietin; N-epsilon-acetylhyposialoerythropoietin; and alpha-N-acetyl, N-epsilon-acetylhyposialoerythropoietin.
36. The method of claim 6 or 8, wherein said tissue protective cytokine is an erythropoietin comprising a succinylated lysine residue.
37. The method of claim 36, where said erythropoietin is selected from the group consisting of alpha-N-succinylerythropoietin; N-epsilon-succinylerythropoietin; alpha-N-succinyl, N-epsilon-succinylerythropoietin; alpha-N-succinylalioerythropoietin; N-epsilon-succinylalioerythropoietin; alpha-N-succinyl, N-epsilon-succinylalioerythropoietin; alpha-

N-succinylhyposialoerythropoietin; N-epsilon-succinylhyposialoerythropoietin; and alpha-N-succinyl, N-epsilon-succinylhyposialoerythropoietin.

38. The method of claim 6 or 8, wherein said tissue protective cytokine is an erythropoietin with at least one lysine residue modified by a 2, 4, 6-trinitrobenzenesulfonic acid salt.
39. The methods of claim 38, wherein the salt is 2, 4, 6-trinitrobenzenesulfonate sodium.
40. The method of claim 6 or 8, wherein said tissue protective cytokine is an erythropoietin in which at least one tyrosine residue is nitrated and/or iodinated.
41. The method of claim 6 or 8, wherein said tissue protective cytokine is an erythropoietin in which an aspartic acid and/or glutamic acid residue is reacted with a carbodiimide followed by reaction with an amine.
42. The method of claim 41, wherein said amine is glycynamide.
43. The method of claim 6 or 8, wherein the inflammation results from a disease condition or trauma.
44. The method of claim 43, wherein the trauma is selected from the group consisting of angitis, chronic bronchitis, pancreatitis, osteomyelitis, rheumatoid arthritis, glomerulonephritis, optic neuritis, temporal arteritis, encephalitis, meningitis, transverse myelitis, dermatomyositis, polymyositis, necrotizing fasciitis, hepatitis, and necrotizing enterocolitis.
45. The method of claim 6 or 8, wherein the tissue protective cytokine inhibits inflammation resulting from cytokines produced by glial cells.
46. The method of claim 6 or 8, wherein the inflammation is triggered by apoptosis.
47. Use of a tissue protective cytokine for the preparation of a pharmaceutical composition for treating inflammation in a mammal comprising responsive cells, tissues, and/or organs.

48. The use of Claim 47, wherein the tissue protective cytokine lacks at least one activity selected from the group consisting of increasing hematocrit, vasoconstriction, hyperactivating platelets, pro-coagulant activity, and increasing production of thrombocytes.
49. The use of claim 47, wherein the inflammation results from a disease condition or trauma.
50. The use of claim 49, wherein the trauma is caused by a seizure disorder, multiple sclerosis, stroke, hypotension, cardiac arrest, ischemia, myocardial infarction, age-related loss of cognitive function, radiation damage, cerebral palsy, neurodegenerative disease, Alzheimer's disease, Parkinson's disease, Leigh disease, AIDS dementia, memory loss, amyotrophic lateral sclerosis, alcoholism, mood disorder, anxiety disorder, attention deficit disorder, autism, Creutzfeld-Jakob disease, brain trauma, spinal cord trauma, brain ischemia, spinal cord ischemia, heart-lung bypass, chronic heart failure, macular degeneration, diabetic neuropathy, diabetic retinopathy, glaucoma, retinal ischemia, or retinal trauma.
51. Use of a tissue protective cytokine for the preparation of a pharmaceutical composition for treating inflammation in a mammal comprising responsive cells, tissues, and/or organs, wherein the pharmaceutical composition comprises a therapeutically effective amount of a tissue protective cytokine; at least one anti-inflammatory agent or immunomodulatory agent; and a pharmaceutically acceptable carrier.
52. The use of claim 47, wherein said tissue protective cytokine is i) an erythropoietin that lacks sialic acid moieties; ii) an erythropoietin that lacks N-linked or lacks O-linked carbohydrates; iii) an erythropoietin having a reduced carbohydrate content by treatment of native erythropoietin with at least one glycosidase; iv) an erythropoietin having at least one or more oxidized carbohydrates; v) an erythropoietin comprising at least one or more oxidized carbohydrates which is chemically reduced; vi) an erythropoietin comprising at least one or more modified arginine residues; vii) an erythropoietin comprising at least one or more modified

lysine residues or a modification of the N-terminal amino group of the erythropoietin molecule; viii) an erythropoietin comprising at least a modified tyrosine residue; ix) an erythropoietin comprising at least a modified aspartic acid or a glutamic acid residue; x) an erythropoietin comprising at least a modified tryptophan residue; xi) an erythropoietin having at least one amino group removed; xii) an erythropoietin comprising at least an opening of at least one of the cystine linkages in the erythropoietin molecule; and xiii) a truncated erythropoietin.

1/29

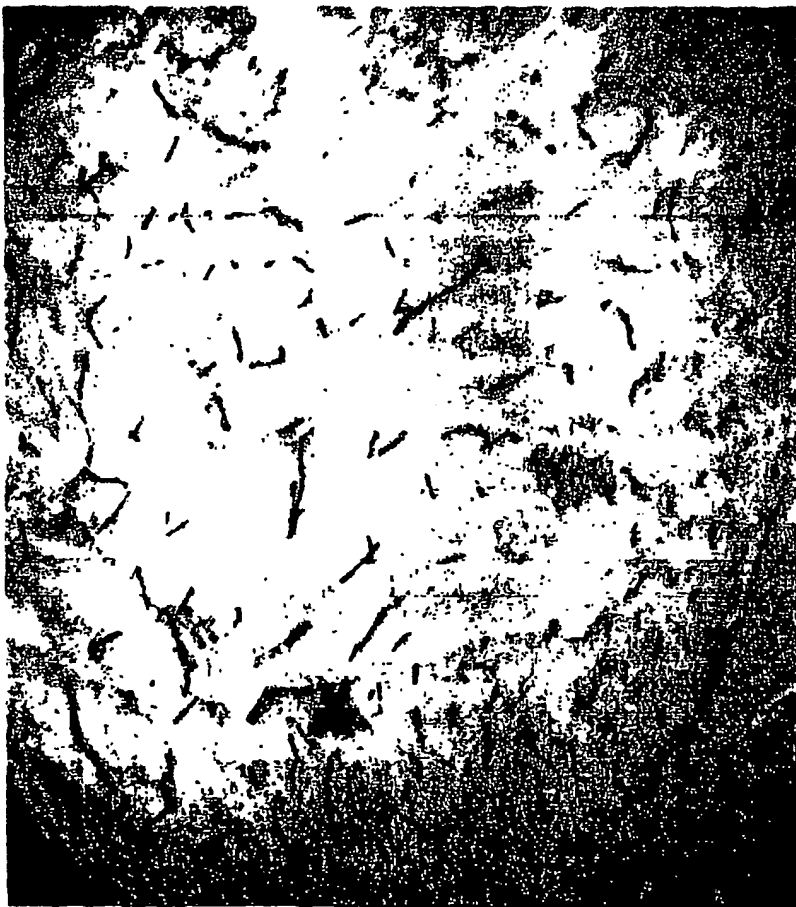


FIG. 1

2/29

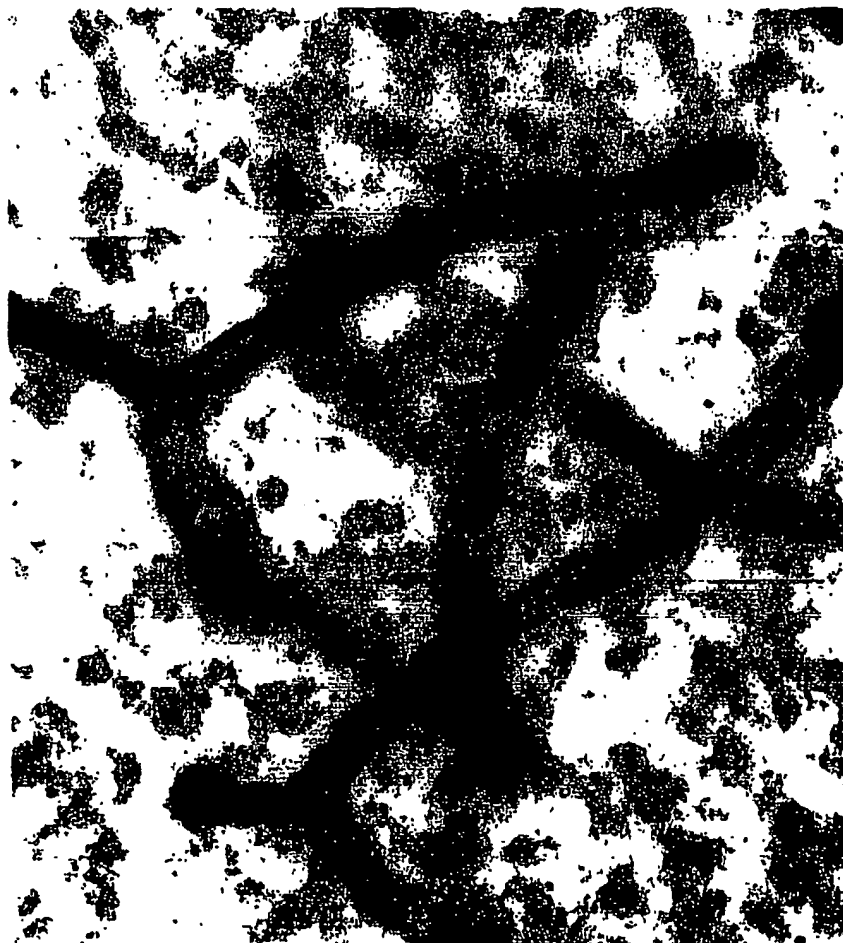


FIG.2

3/29



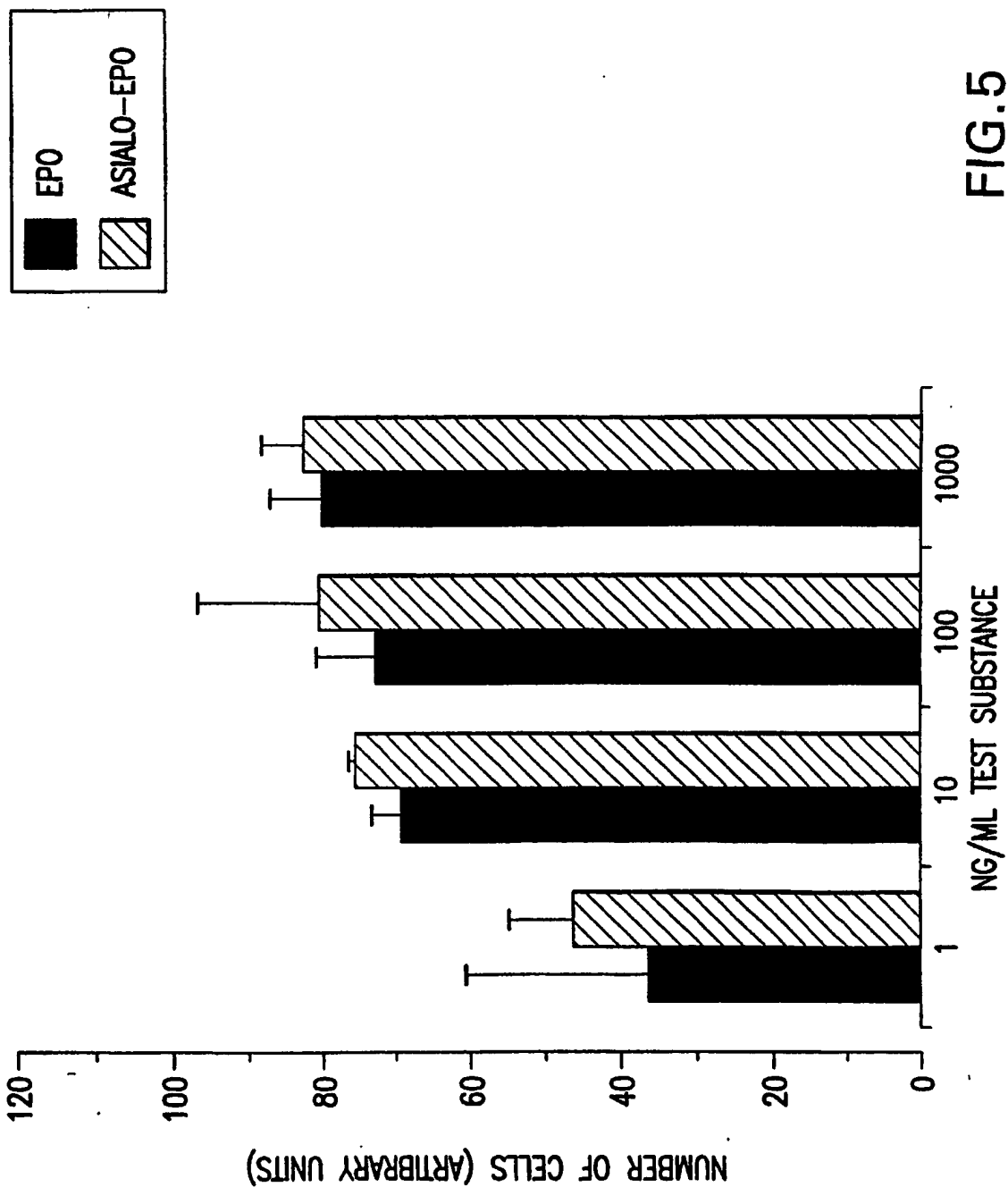
FIG. 3

4/29



FIG. 4

5/29



6/29

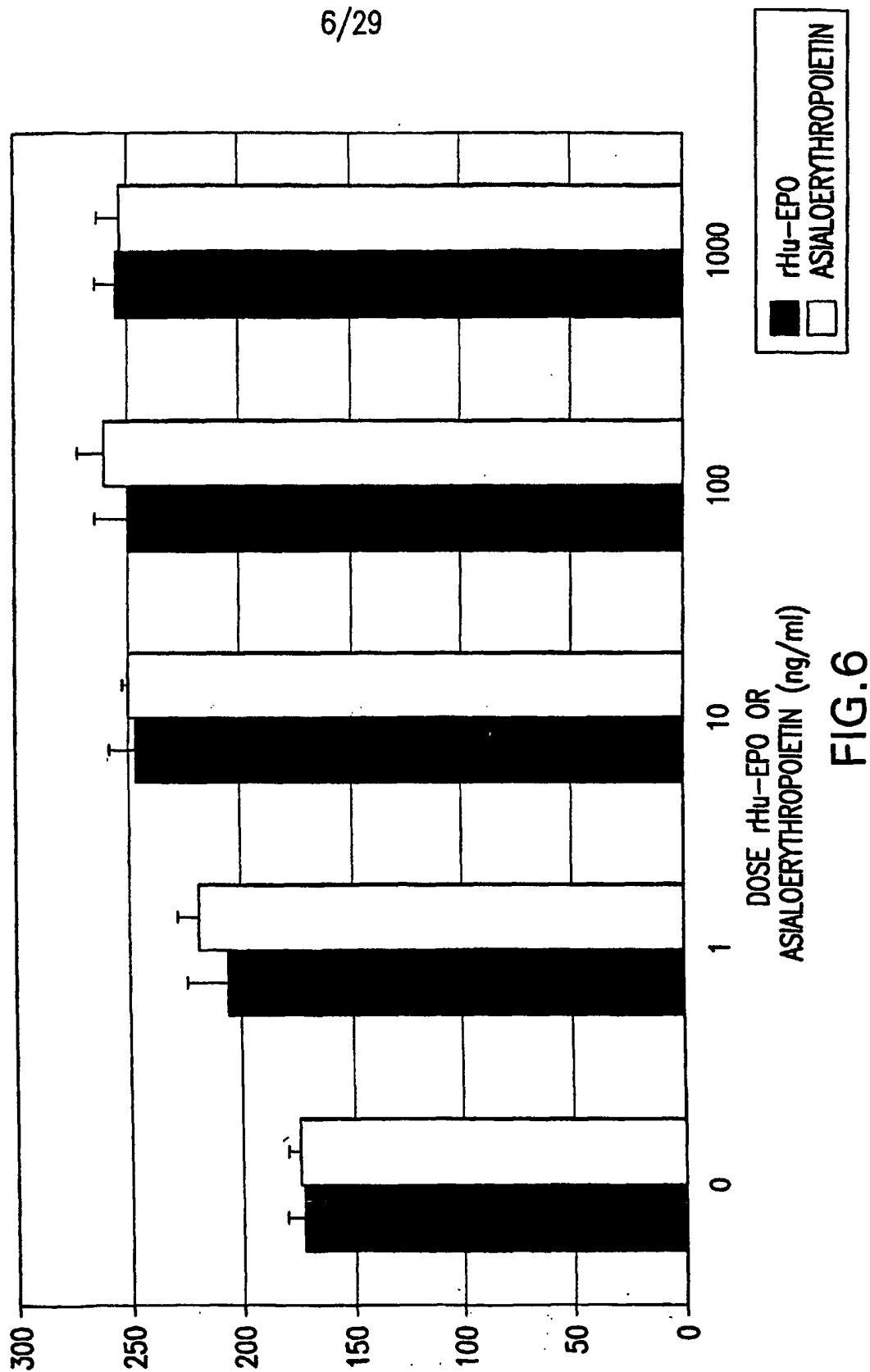


FIG. 6

7/29

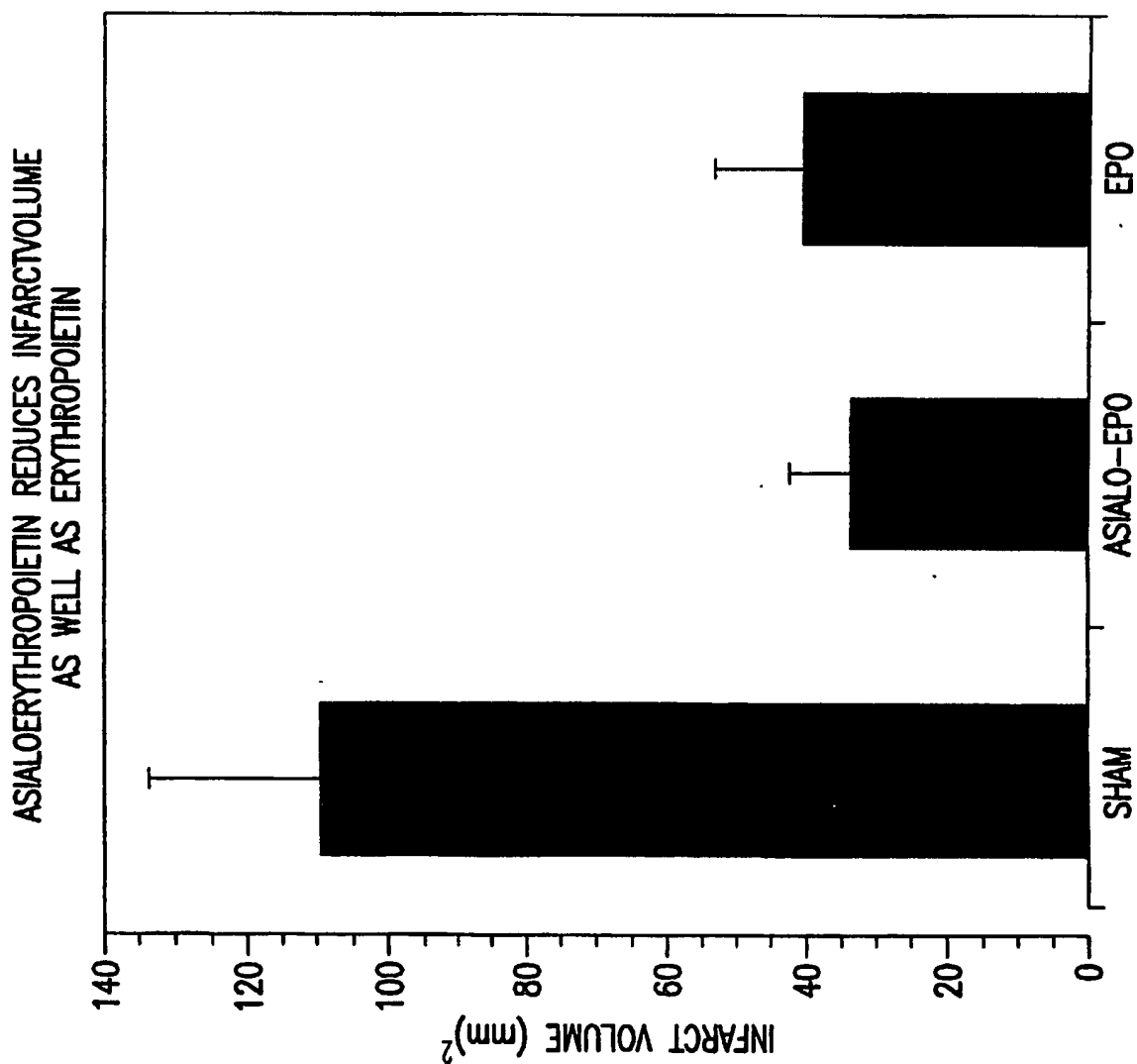
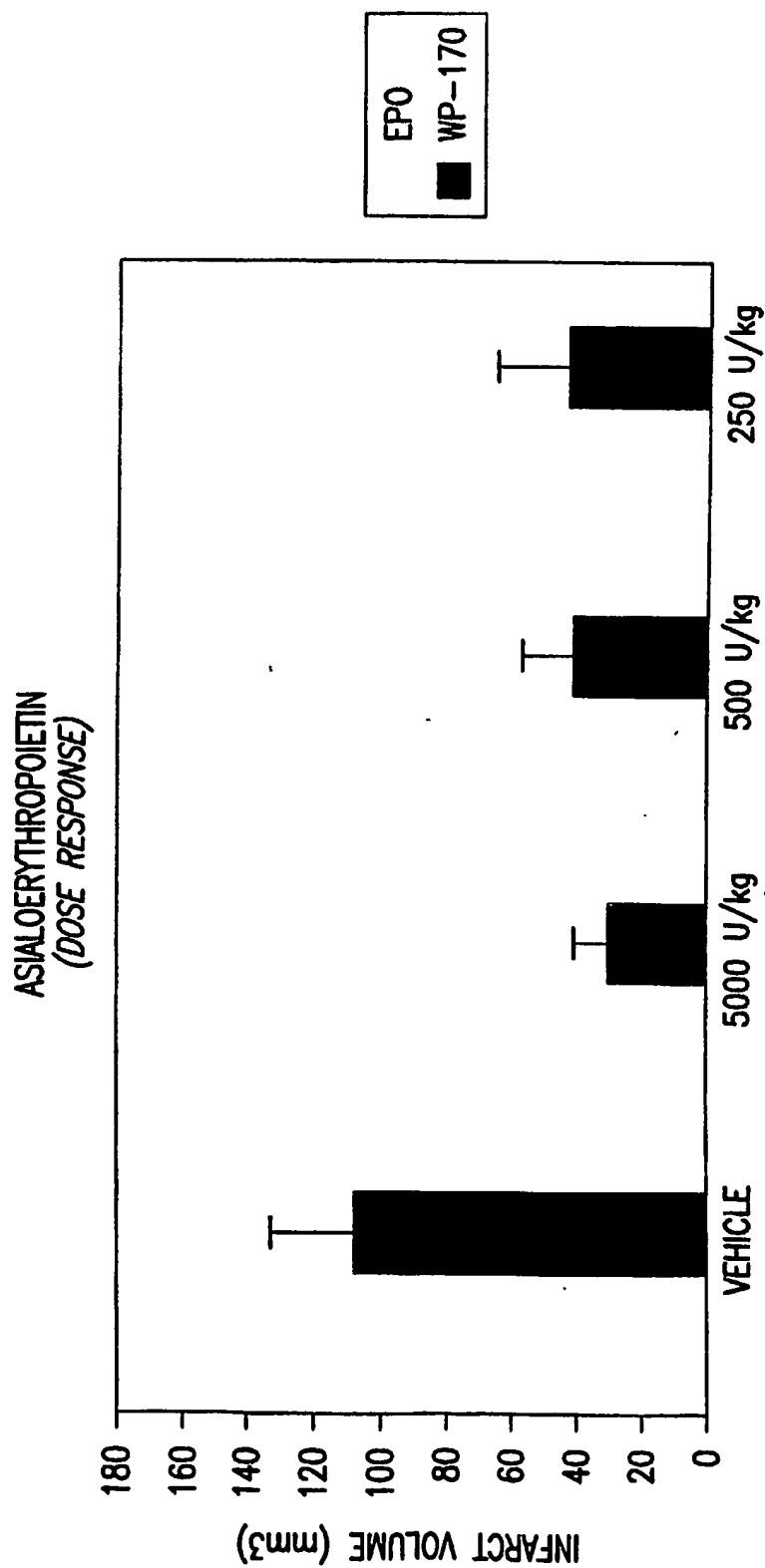


FIG. 7

8/29



**FIG.8**

n FOR EACH GROUP IS GREATER  
THAN OR EQUAL TO 4

9/29

PROTECTION OF P19 CELLS FROM SERUM  
DEPRIVATION BY IODO-EPO

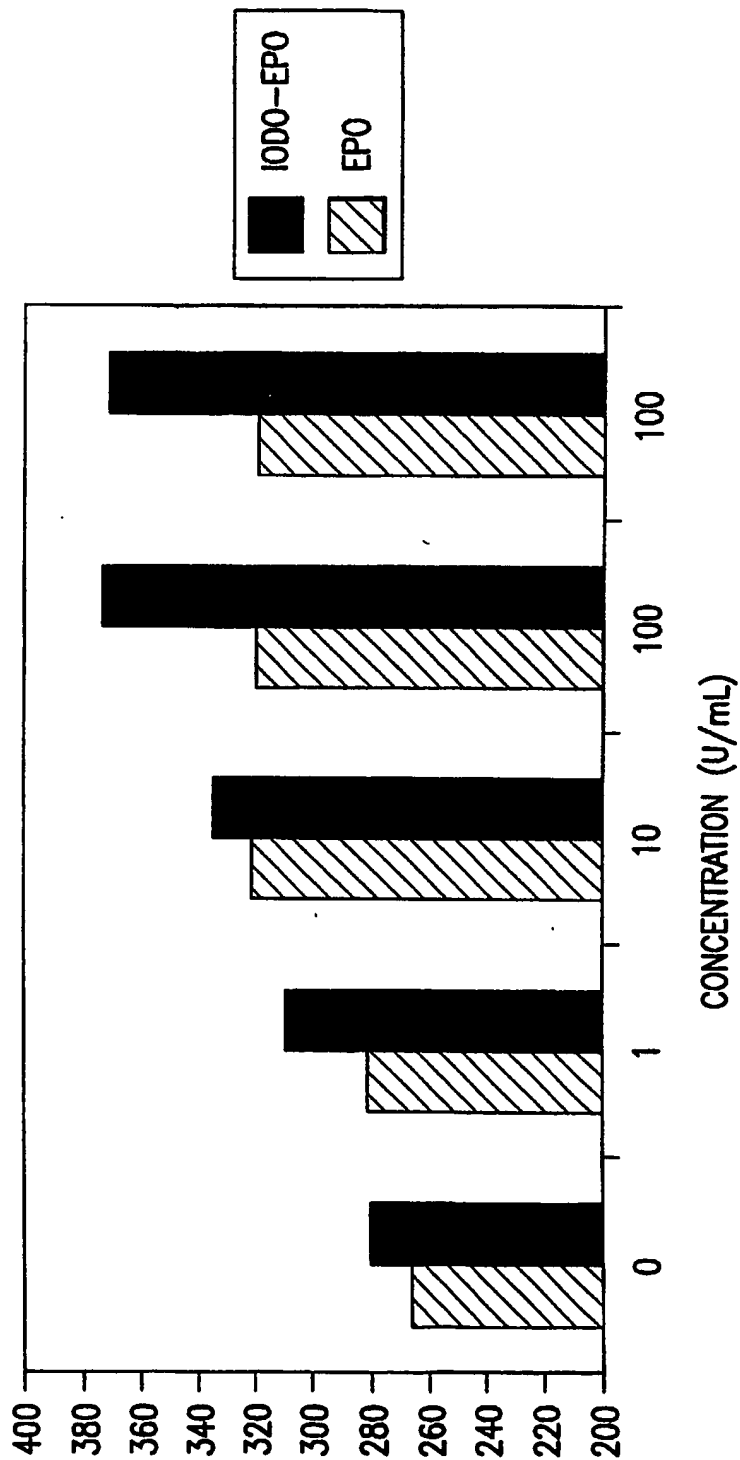


FIG.9

10/29

BIOTINYLATED-EPO AND ASIALO-EPO RETAIN  
ACTIVITY IN P19 ASSAY

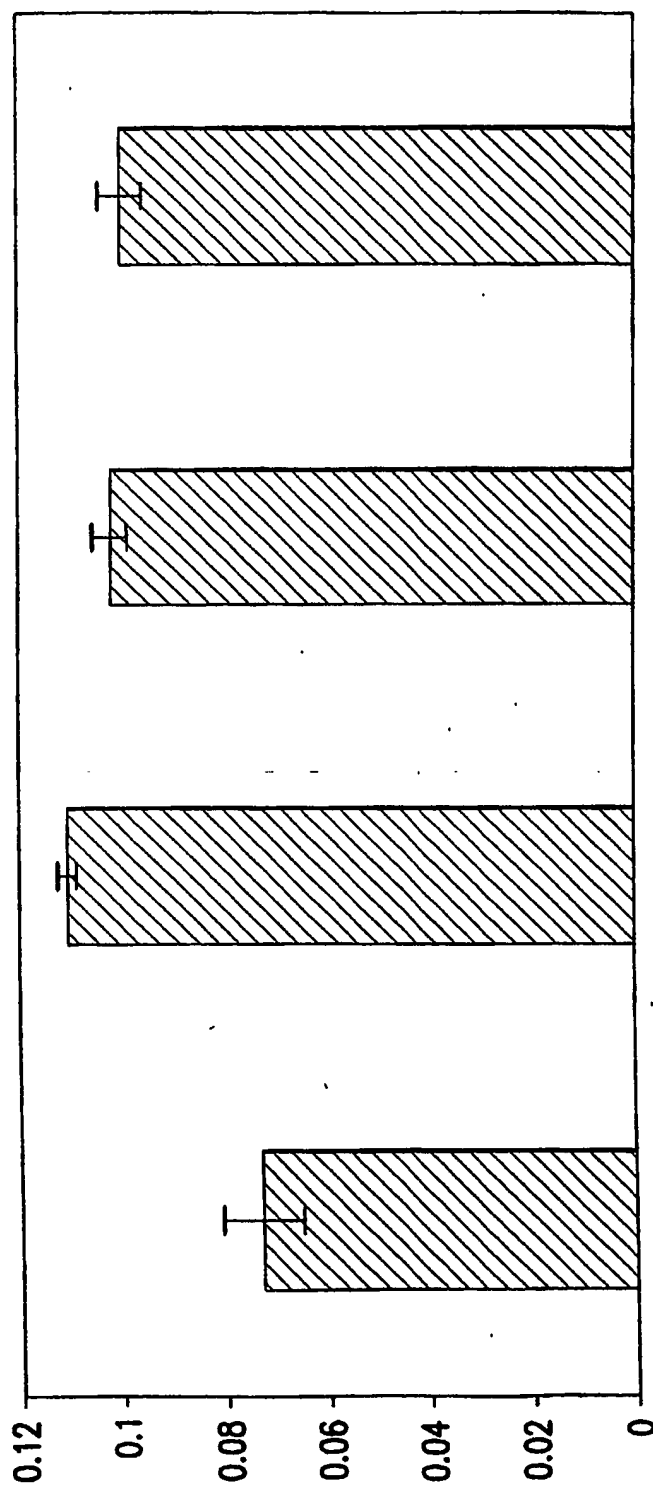
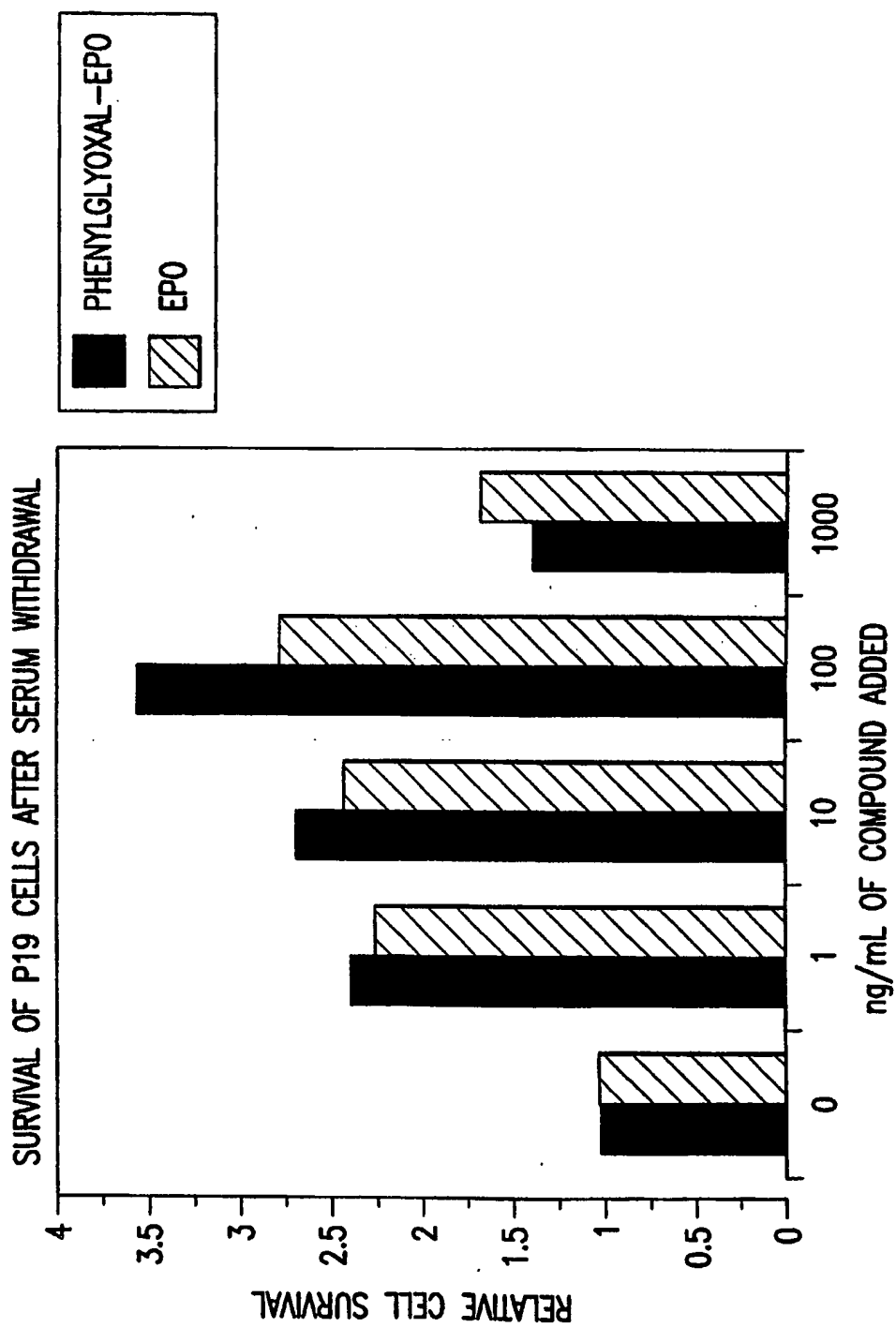


FIG.10

11/29



12/29

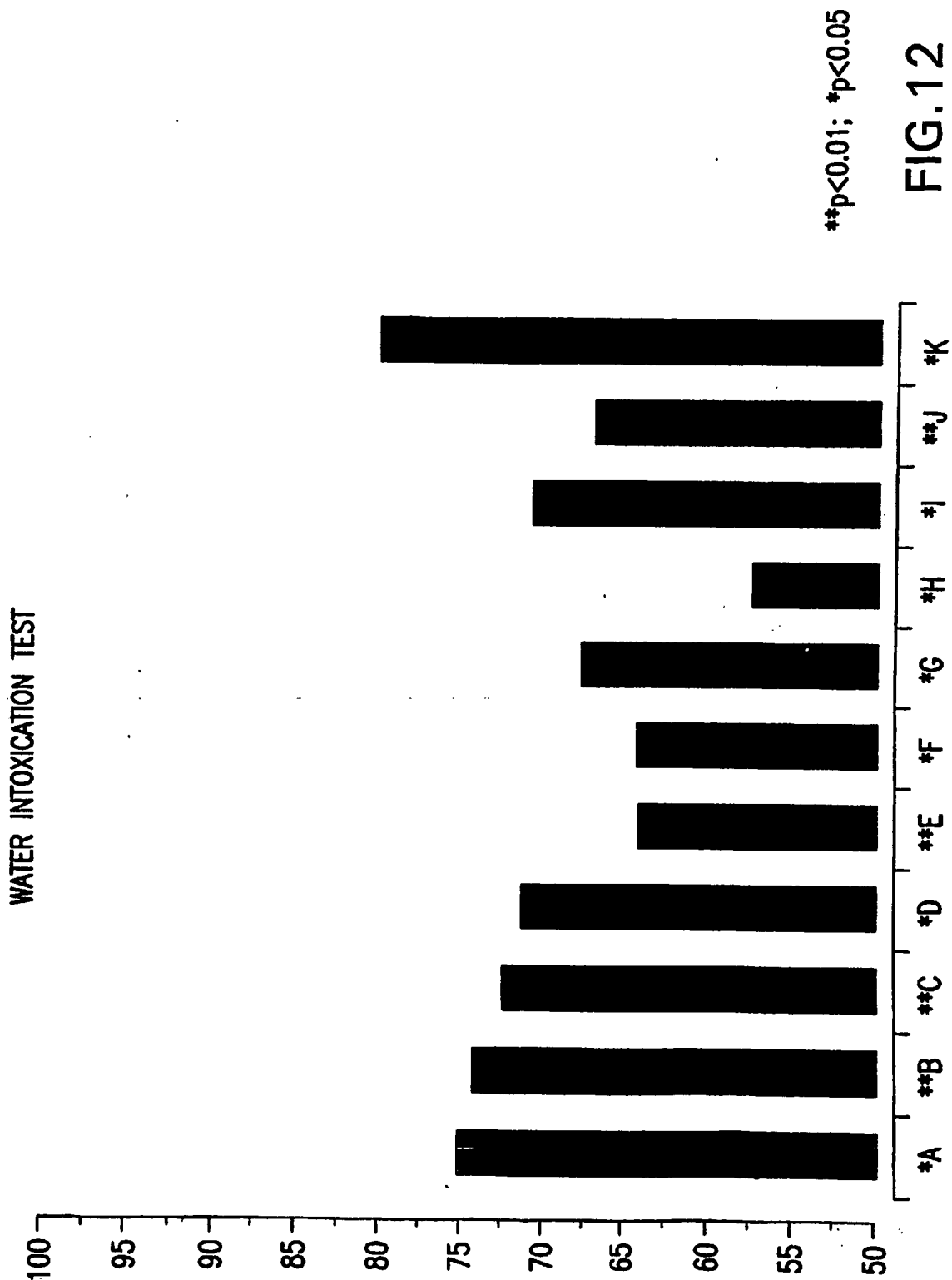


FIG.12

13/29

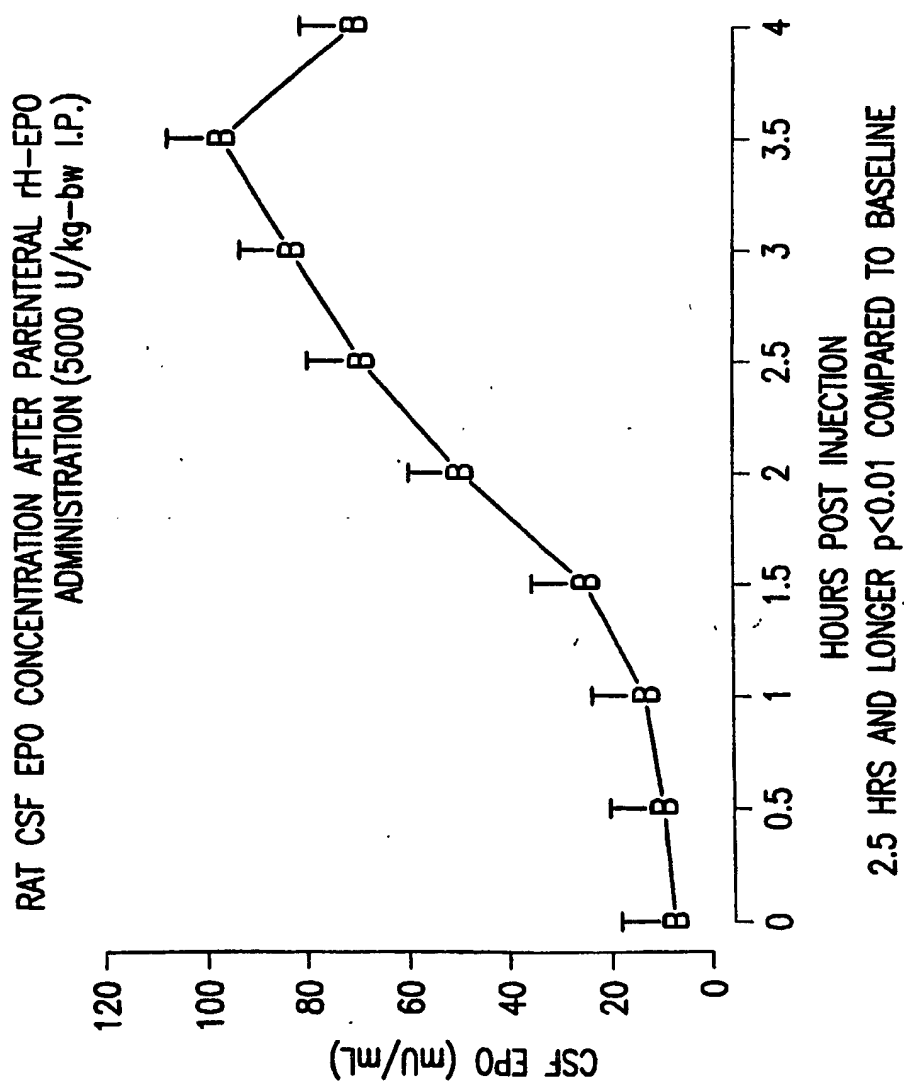


FIG.13

14/29

ERYTHROPOIETIN IMPROVES CARDIAC FUNCTION IN  
A HEART ISOLATED FOR TRANSPLANTATION

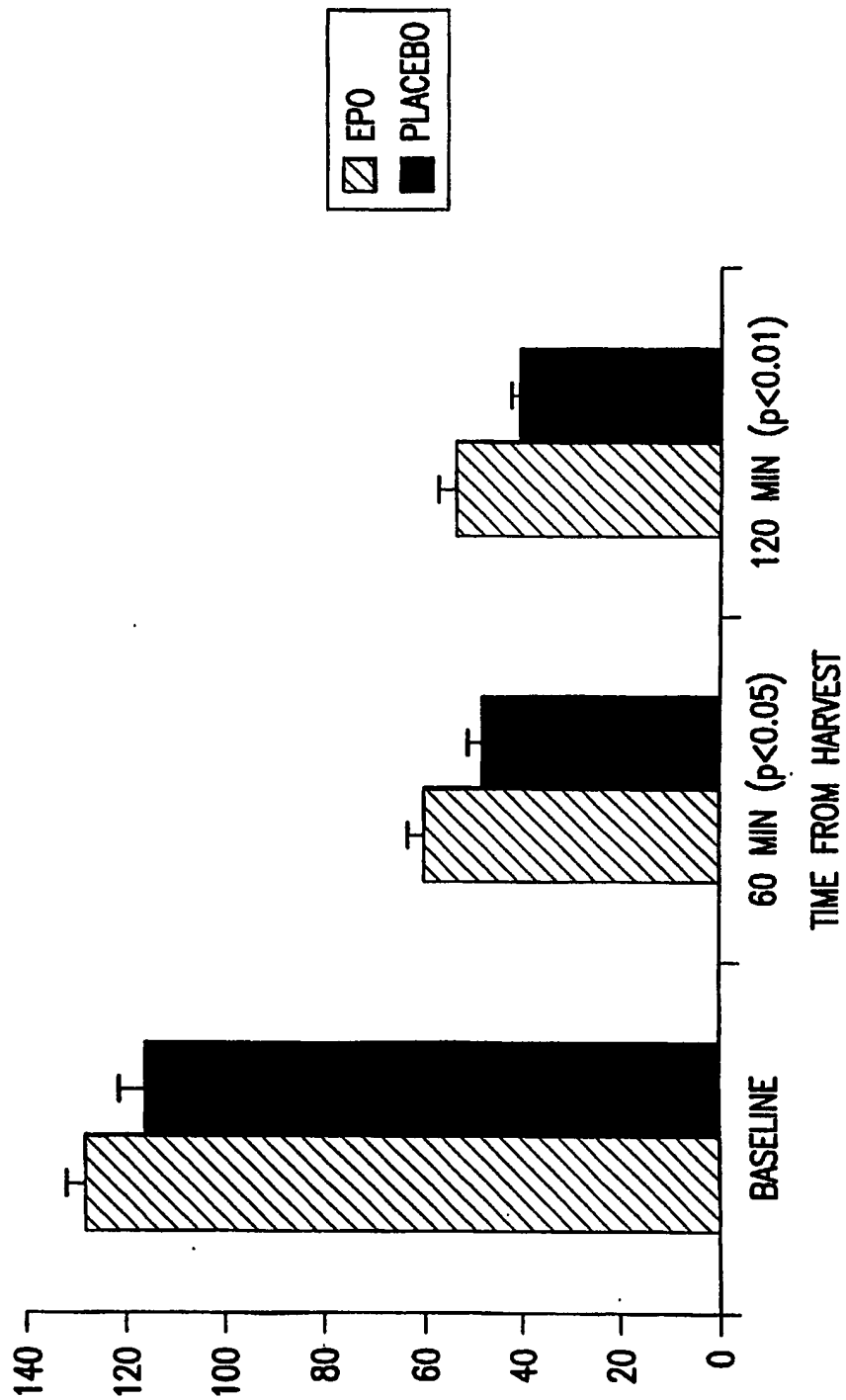
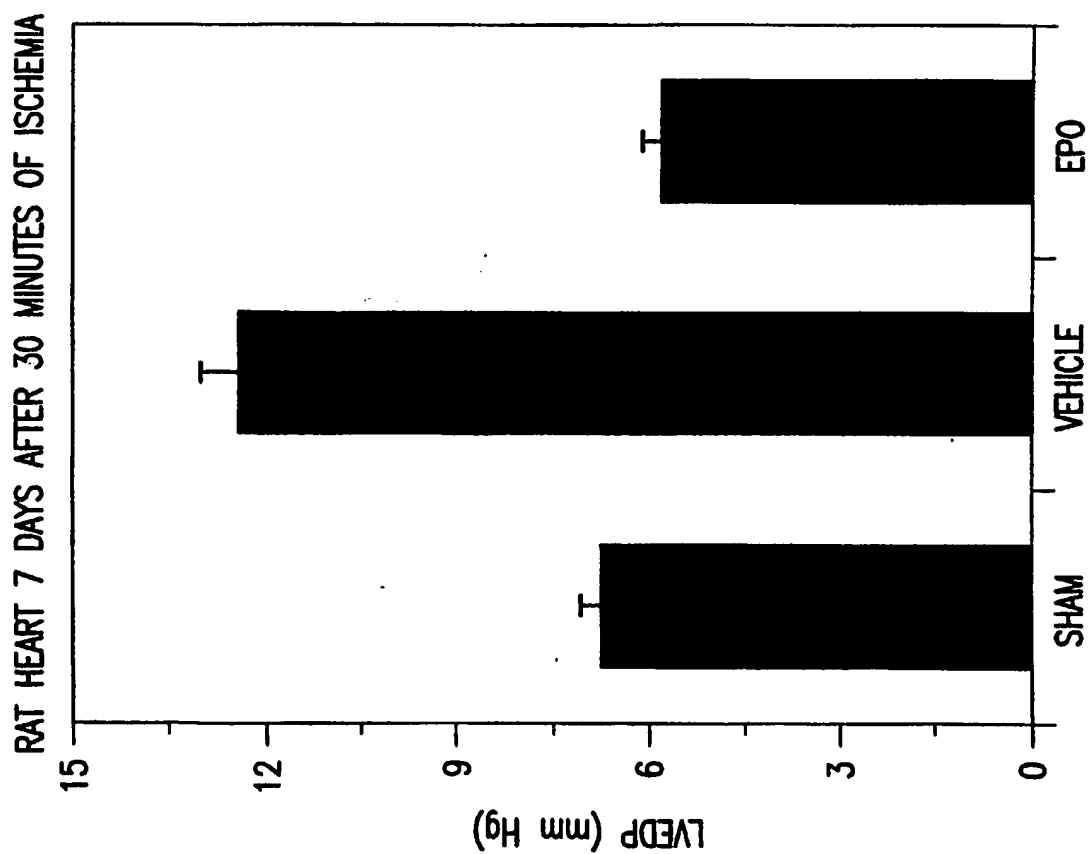


FIG. 14

15/29

FIG. 15



ELECTRORETINOGRAMS  
FROM RATS SUBJECTED  
TO 60 MINUTES  
OF ISCHEMIA

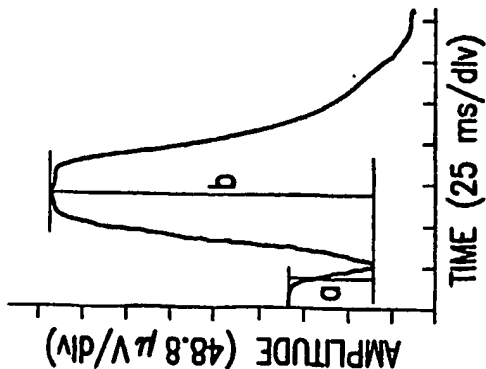


FIG. 16A

ELECTRORETINOGRAMS  
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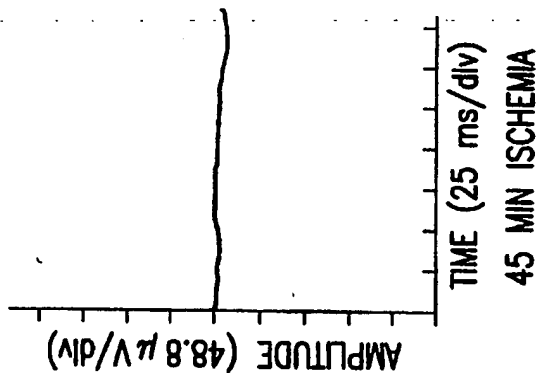


FIG. 16B

ELECTRORETINOGRAMS  
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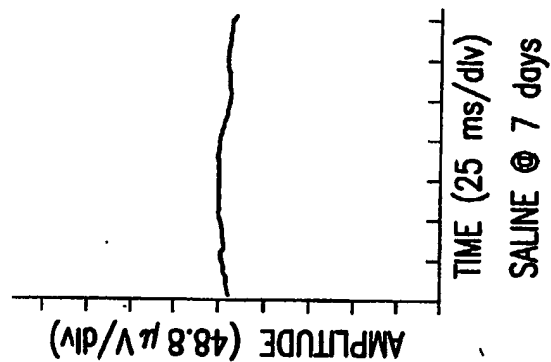


FIG. 16C

ELECTRORETINOGRAMS  
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TO 60 MINUTES  
OF ISCHEMIA

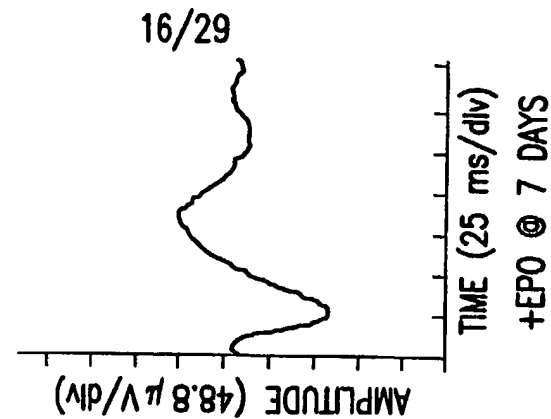


FIG. 16D

17/29

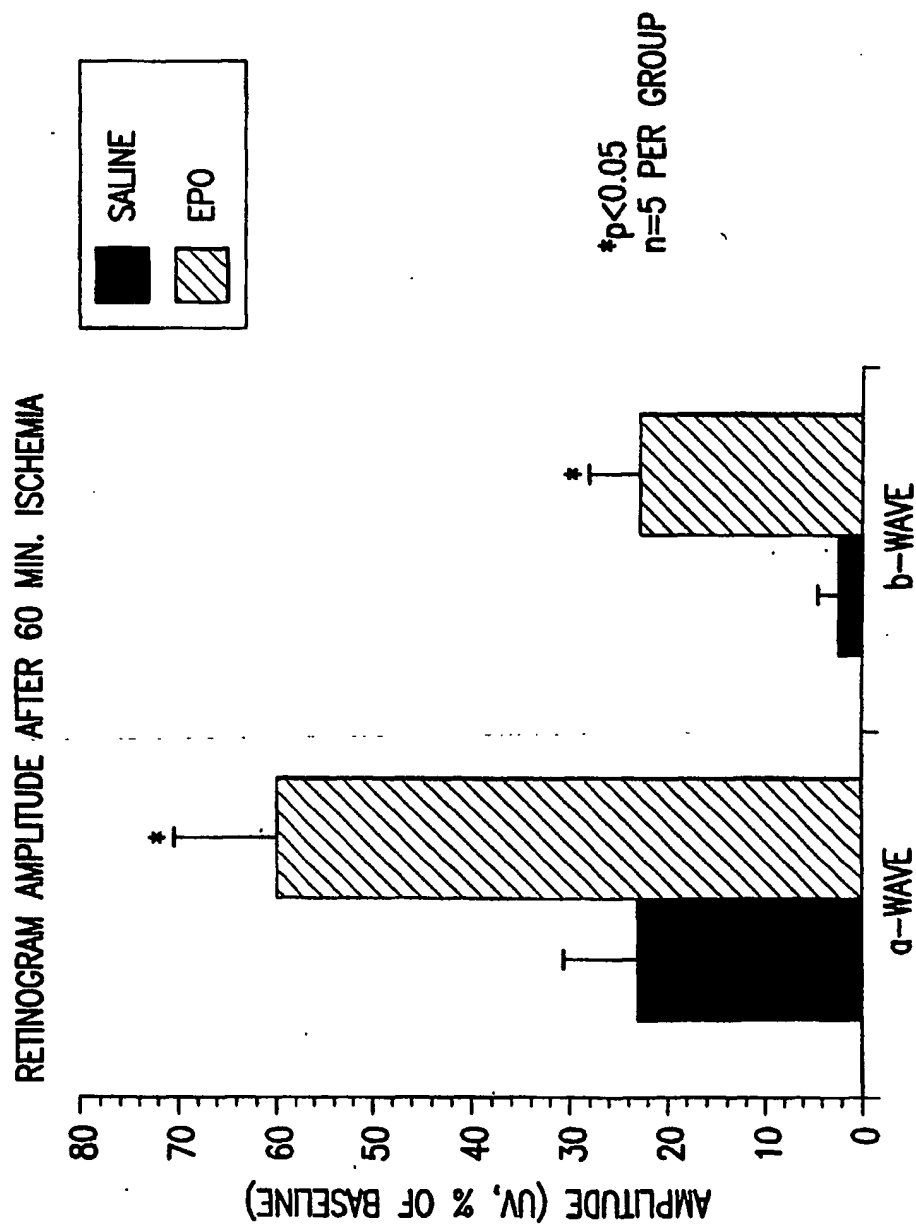


FIG.17

18/29

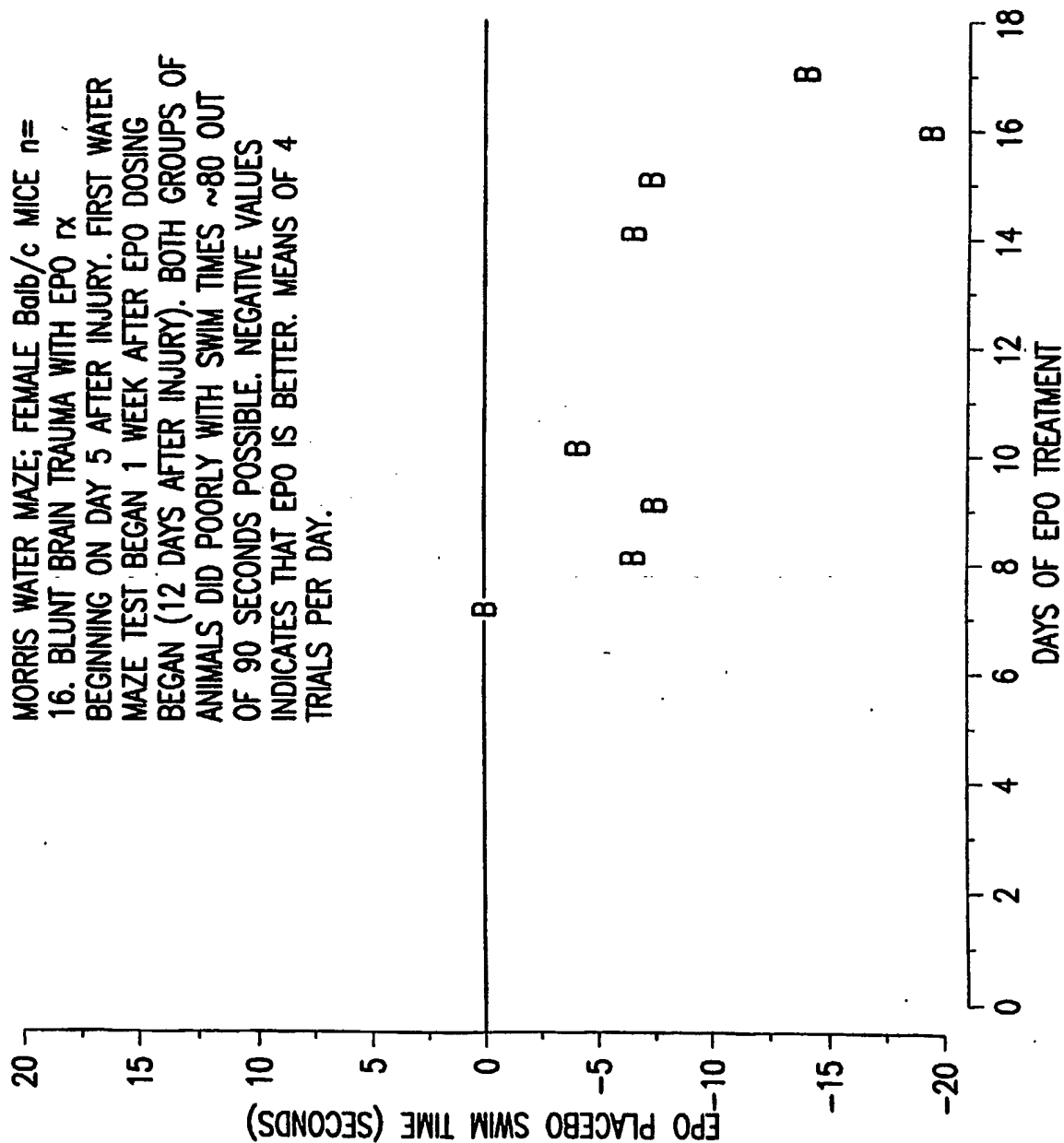


FIG.18

19/29

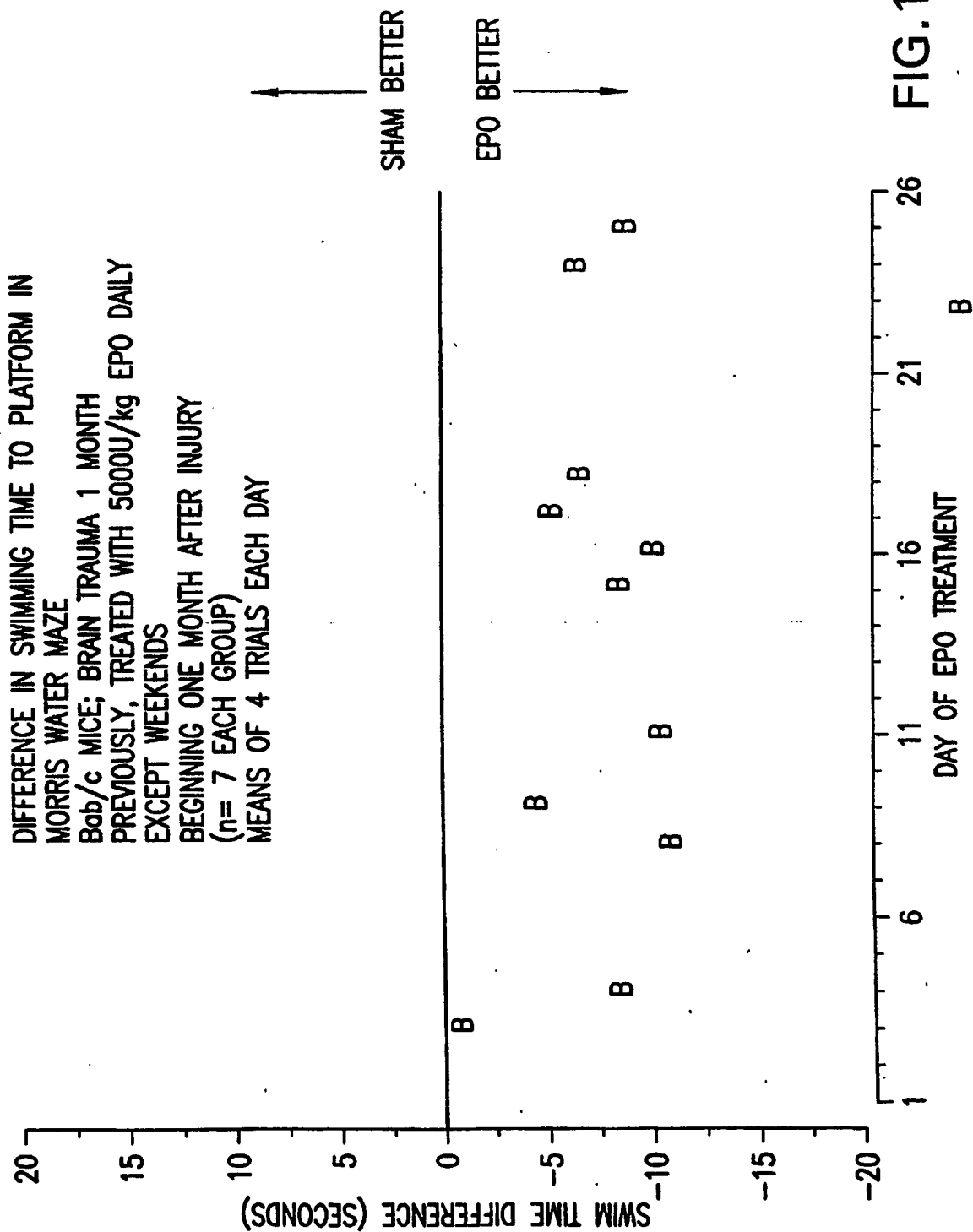


FIG.19

20/29

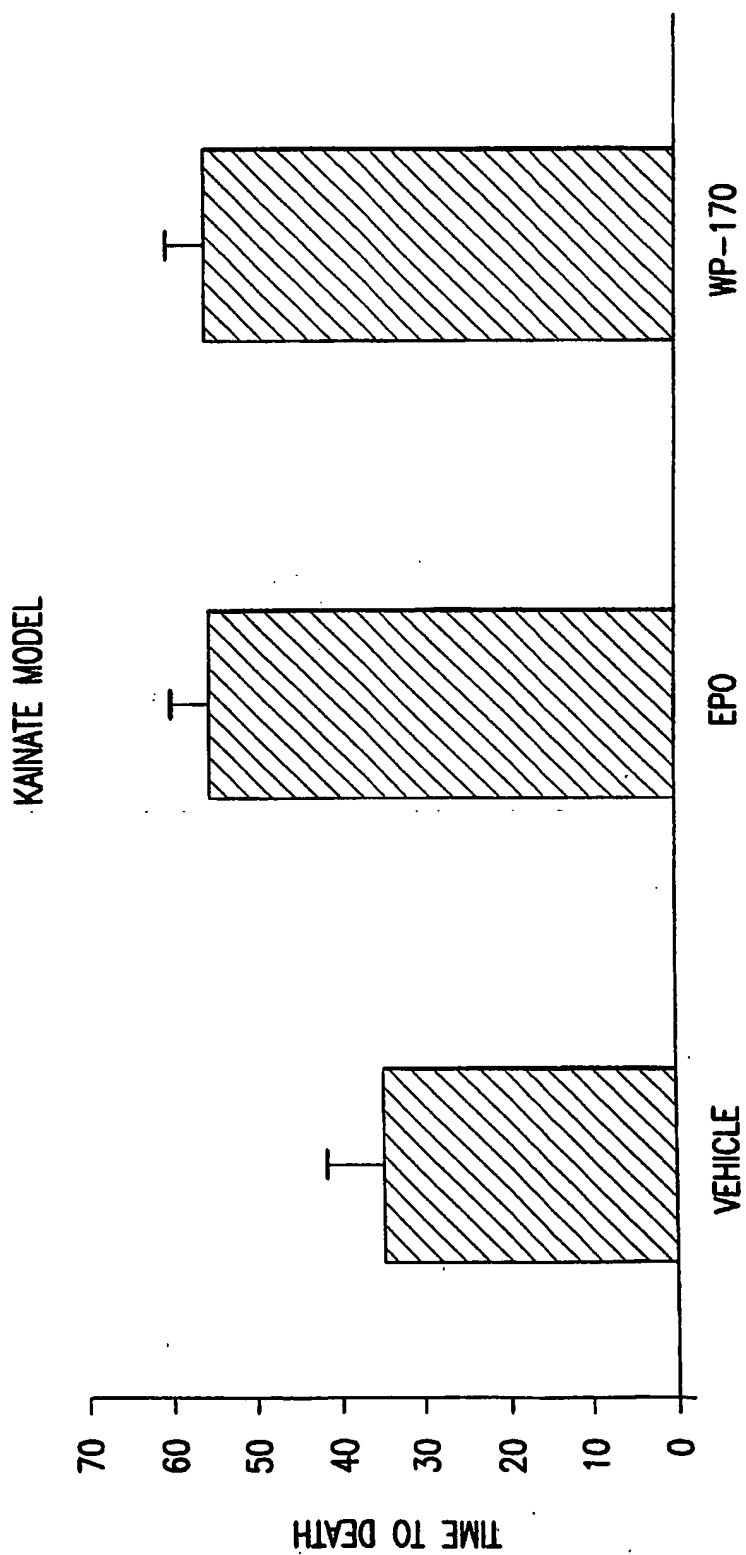


FIG.20

21/29

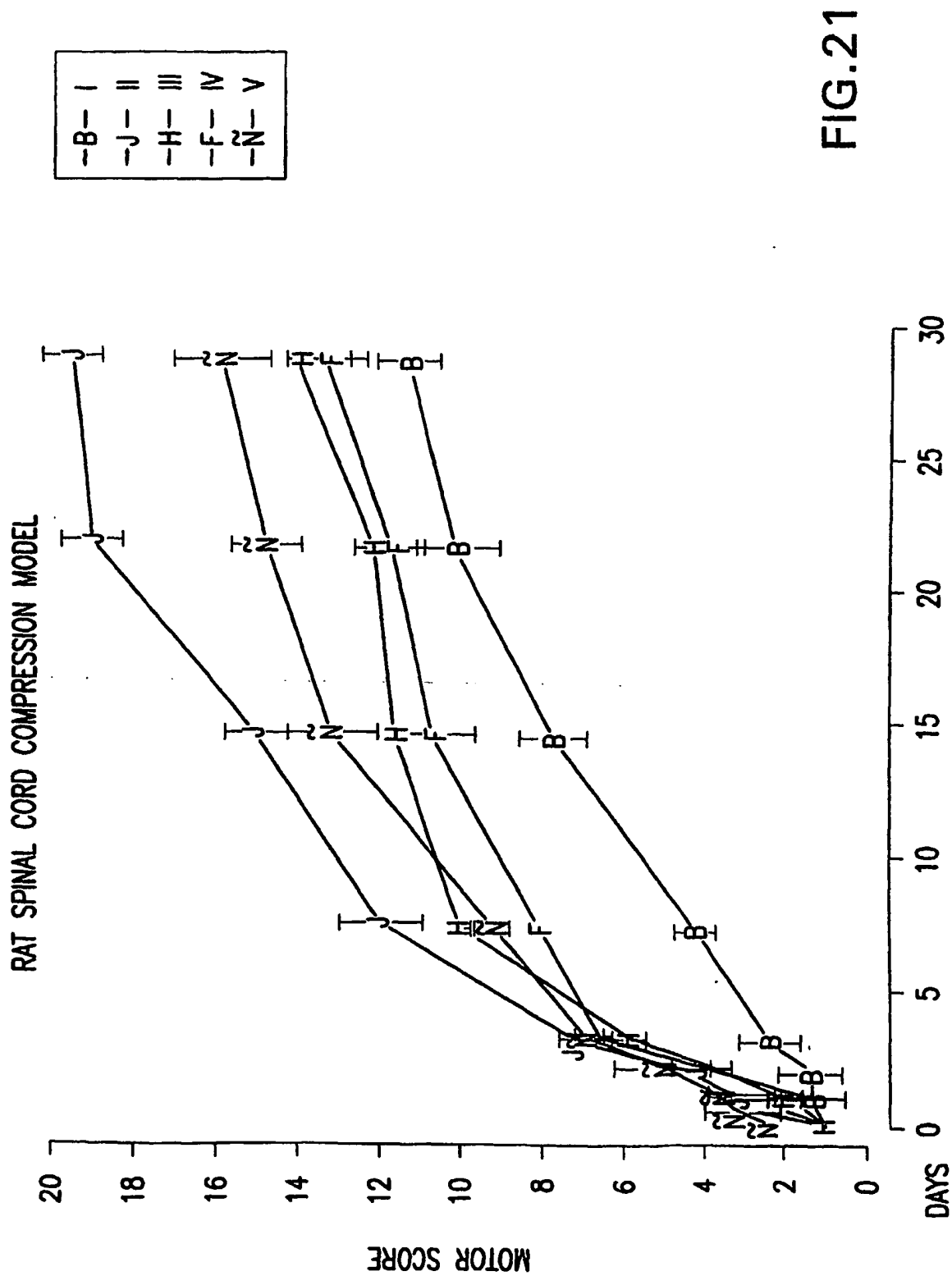


FIG.21

22/29

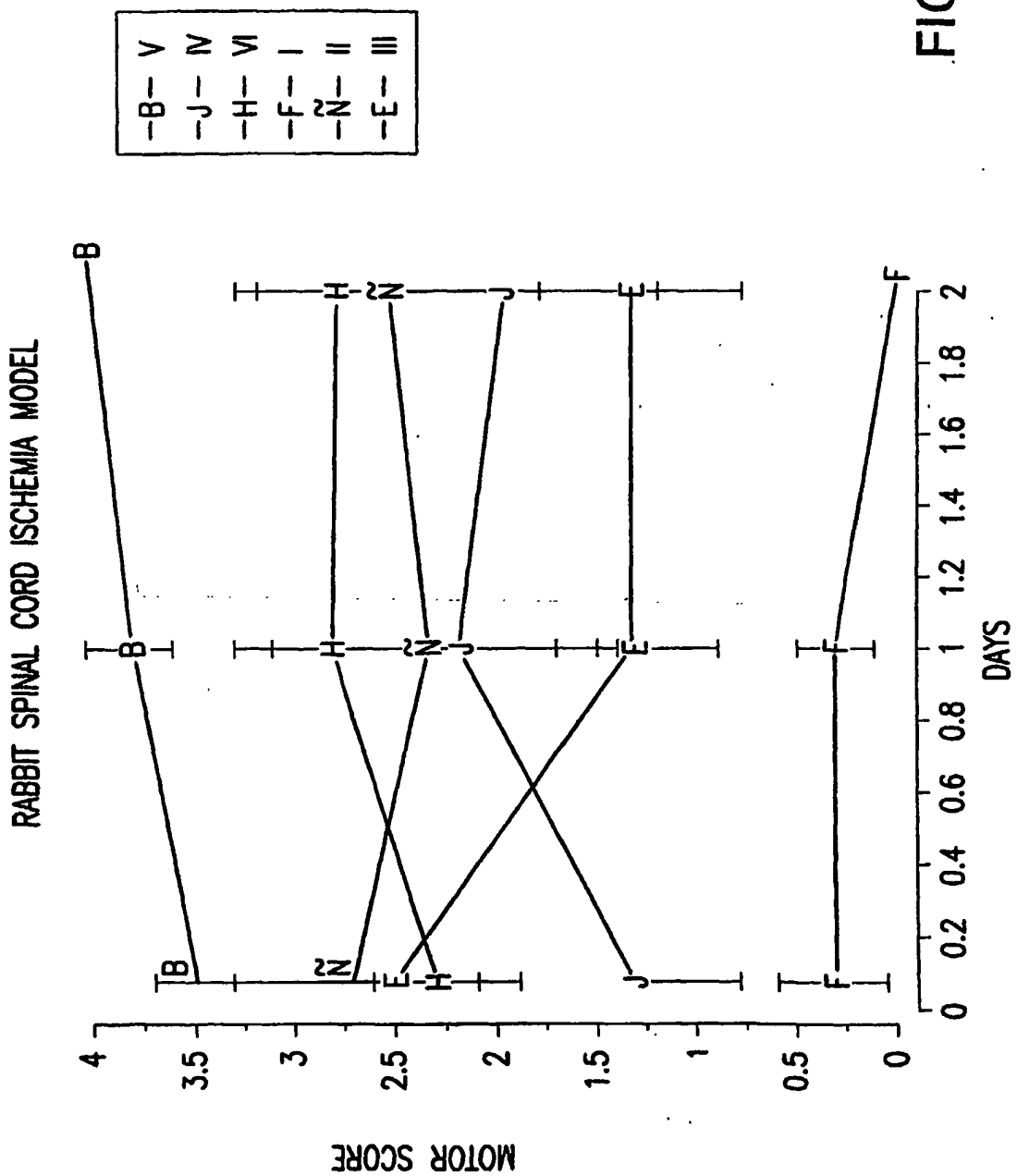


FIG. 22

23/29

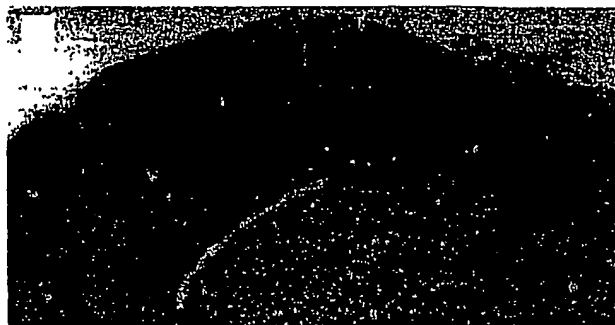


FIG. 23A



FIG. 23B

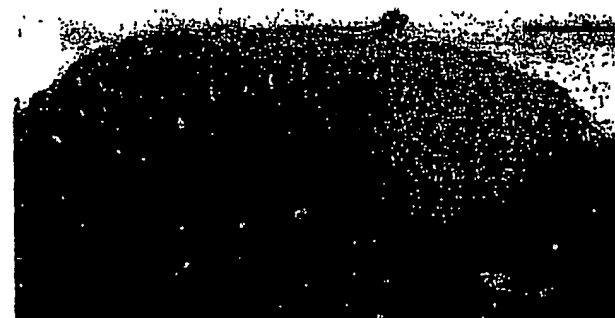


FIG. 23C

24/29

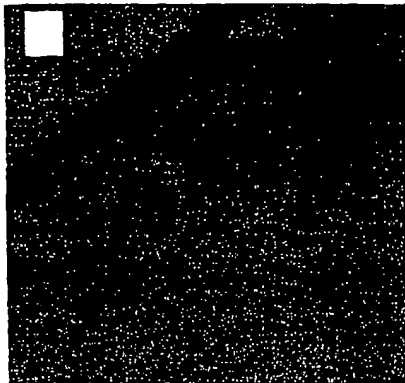


FIG. 24A

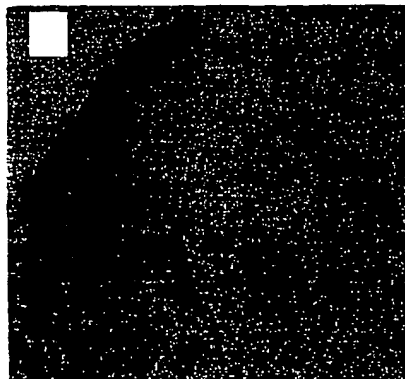


FIG. 24B

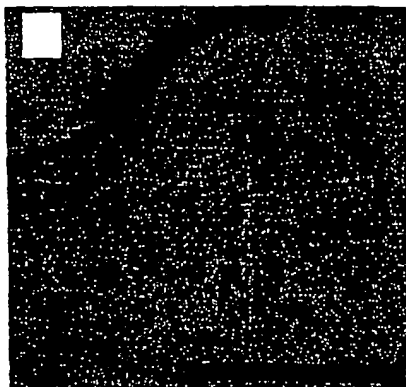


FIG 24C

25/29

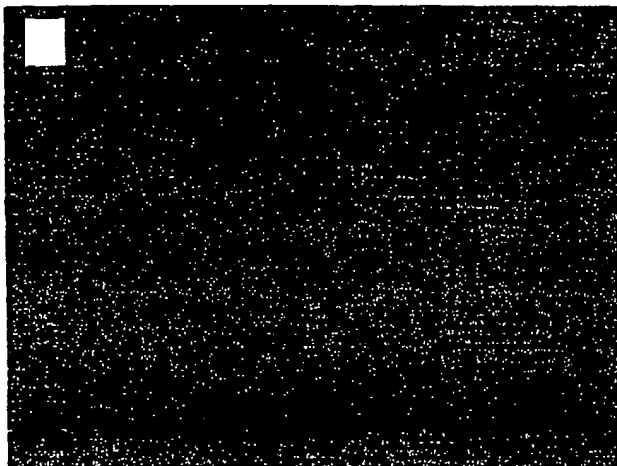


FIG.25A

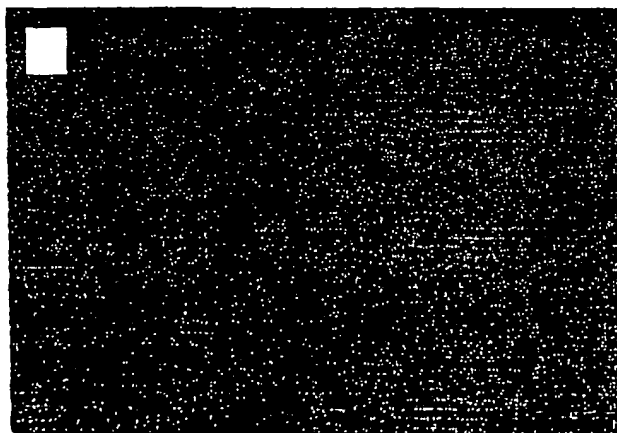


FIG.25B

26/29

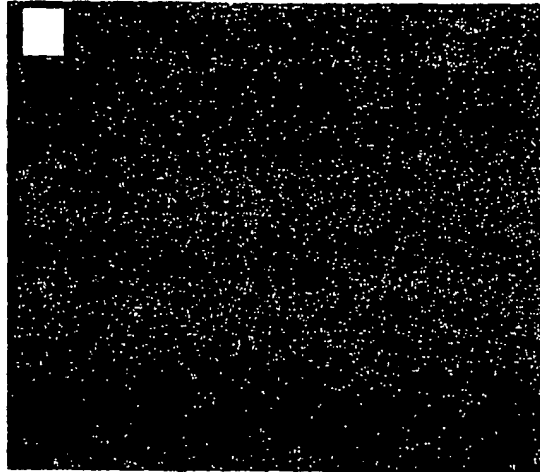


FIG.26A

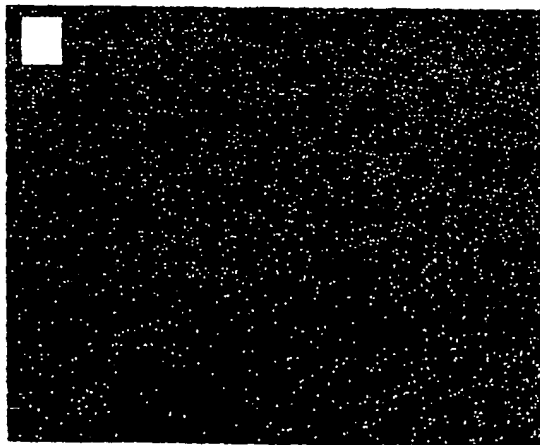


FIG.26B

27/29

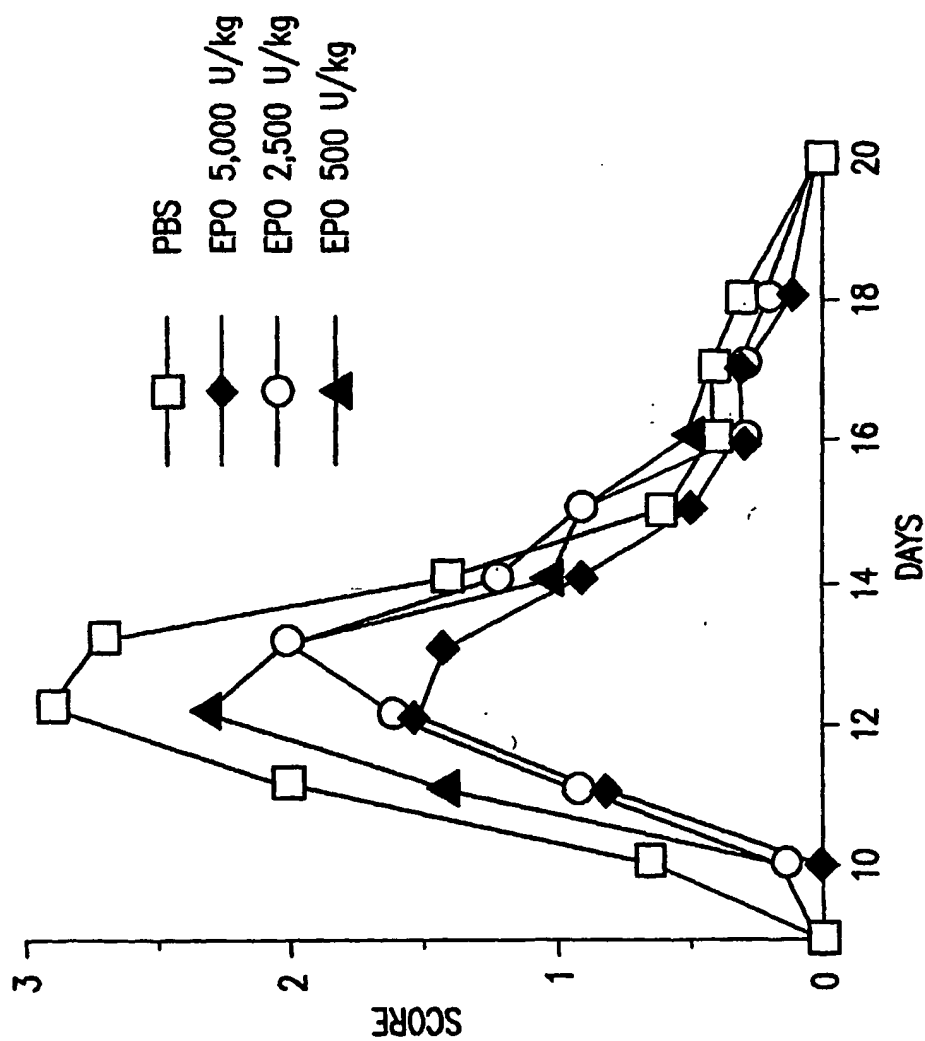


FIG.27

28/29

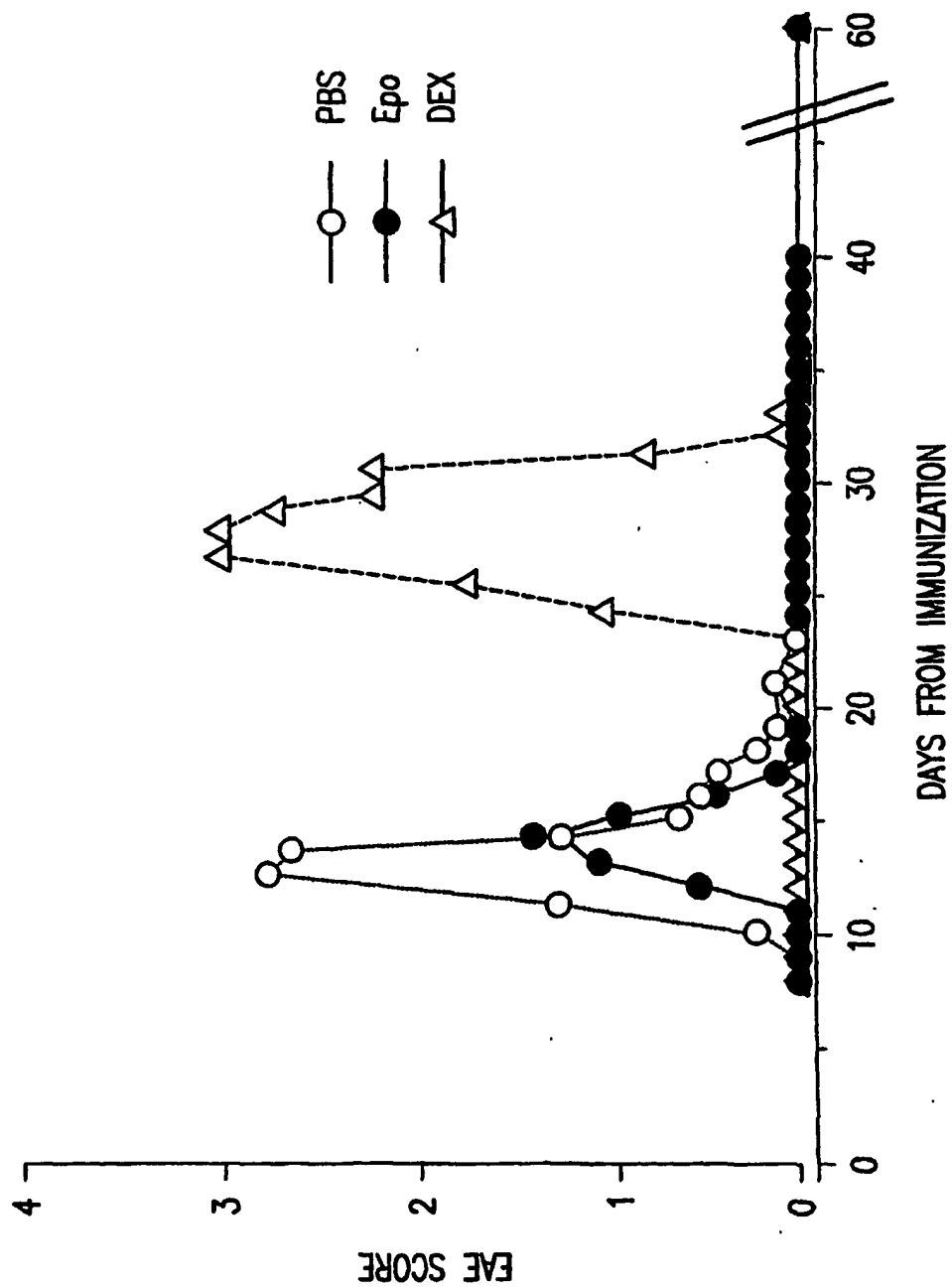


FIG.28

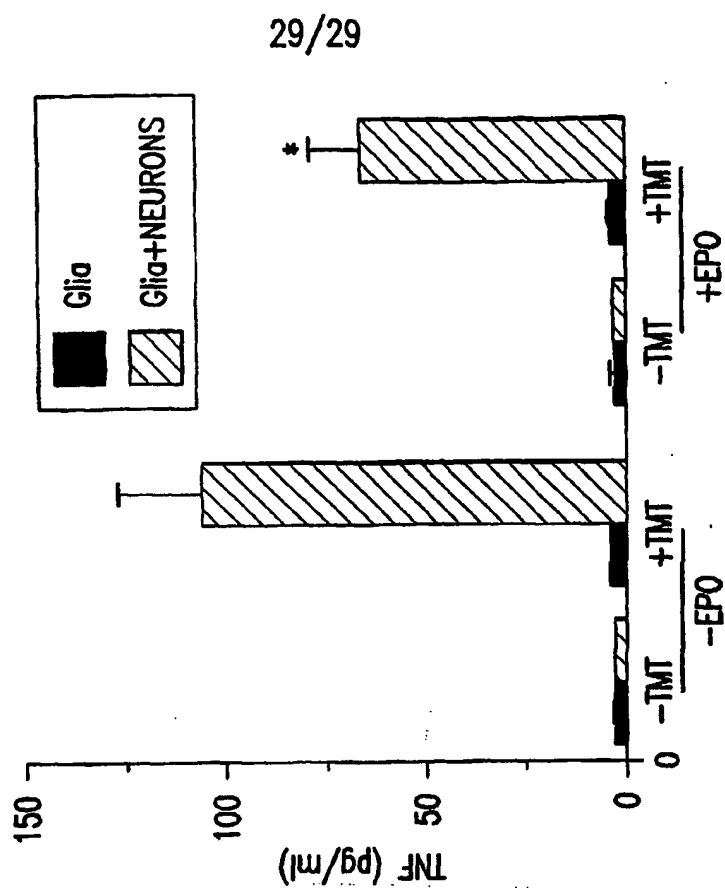


FIG.29B

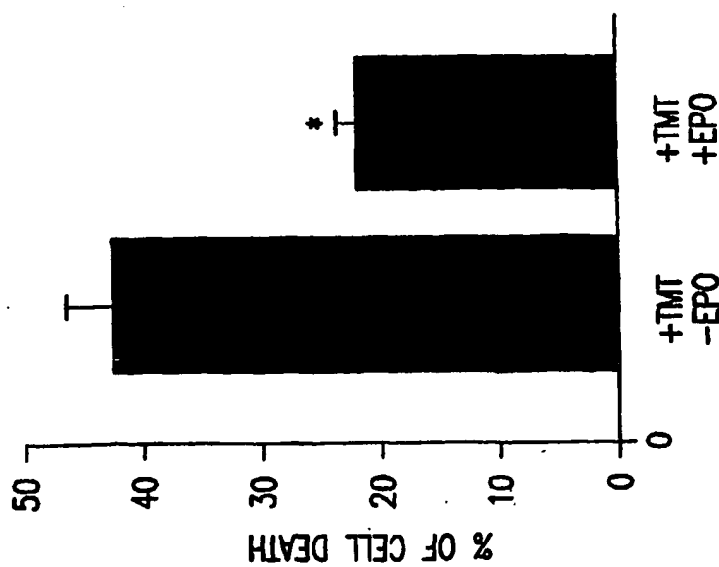


FIG.29A

## SEQUENCE LISTING

<110> The Kenneth S. Warren Institute, Inc.

<120> TISSUE PROTECTIVE CYTOKINES FOR THE PROTECTION, RESTORATION, AND  
ENHANCEMENT OF RESPONSIVE CELLS, TISSUES AND ORGANS

<130> 10165-026-228

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<150> 10/188,905  
<151> 2002-07-03

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<170> PatentIn version 3.0

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Ser Asn Phe Leu Arg Gly

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5

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A61K 45/06 (2006.01) A61K 38/00 (2006.01)

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PCT/US2003/021350

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(26) Publication Language: English

(30) Priority Data:

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(81) Designated States (national): AE, AG, AL, AM, AT, AU,  
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU,  
CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH,  
GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC,  
LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW,  
MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SC, SD, SE,  
SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ,  
VC, VN, YU, ZA, ZM, ZW.

(84) Designated States (regional): ARIPO patent (GH, GM,  
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Burasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ,  
TM), European patent (AT, BE, BG, CH, CY, CZ, DE,  
DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL,  
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CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

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(54) Title: TISSUE PROTECTIVE CYTOKINES FOR THE PROTECTION, RESTORATION, AND ENHANCEMENT OF RE-  
SPONSIVE CELLS, TISSUES AND ORGANS

(57) Abstract: Methods and compositions are provided for treating a mammal having inflammation by protecting or enhancing a  
responsive cell, tissue, organ or body part exhibiting or associated with the inflammation, by systemic or local administration of a  
composition comprising a tissue protective cytokine. The invention also encompasses combination treatments comprising admin-  
istering a composition comprising a tissue protective cytokine of the invention and administering at least one anti-inflammatory or  
least one immunomodulatory agent.

WO 2004/004656 A3

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US03/21350

## A. CLASSIFICATION OF SUBJECT MATTER

IPC: A61K 38/19( 2006.01),45/00( 2006.01)

USPC: 424/85.2,85.1;514/12;530/351

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
U.S. : 424/85.2, 85.1; 514/12; 530/351

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
WEST, US PATENT FULL, STN, VIA MEDLINE, EMBASE, BIOSIS, CAPLUS

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages   | Relevant to claim No. |
|------------|--|-----------------------|
| Y          | IMAI et al. Physiochemical and Biological Characterization of Asialoerythropoietin Suppressive Effects of Sialic Acid in the Expression of Biological Activity of Human Erythropoietin in Vitro. European Journal of Biochemistry. December 1990. Vol. 194, No.2, pages 457-462. | 1-52                  |
| Y          | DONG et al. Receptor Binding of Asialoerythropoietin. Journal of Cellular Biochemistry. March 1992. Vol.48, No.3, pages 269-276  | 1-52                  |
| A          | Yang et al. Effects of Ammonia and Glucoseamine on the Heterogeneity of Erythropoietin Glycoforms. Biotechnology progress January 2002, Vol.18, No.1, pages 129-138  | 1-52                  |

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

### \* Special categories of cited documents:

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